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PRINCIPAL INVESTIGATOR: Terri I. Grodzicker

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**Cold Spring Harbor Laboratory Conference on**
**Microbial Pathogenesis and Host Response**

**September 10-14, 1997**

**305 participants**

**ARRANGED BY:** **P.T. Magee,** ***University of Minnesota***
**Stanley Maloy,** ***University of Illinois***
**Ronald Taylor,** ***Dartmouth Medical School***

Understanding microbial pathogenesis demands a detailed knowledge of the host response as well as the pathogen itself, and requires an interdisciplinary approach, integrating the fields of microbiology, eukaryotic cell biology, and immunology. The first Cold Spring Harbor meeting on Microbial Pathogenesis and Host Response was planned to facilitate such interactions, and the meeting attracted over 300 international scientists who approach the study of bacterial and fungal pathogens from a broad range of perspectives.

Each session focused on recent insights into a specific mechanism of pathogenesis. The first session dealt with bacterial pathogens that produce toxins which cause the primary disease symptoms. A major highlight of this session was a talk by John Mekalanos on how a bacterial virus can convert nonvirulent strains of *Vibrio cholerae* into a highly virulent strain. Another session dealt with *Shigella, Listeria,* and *Legionella*, three bacterial pathogens that evade the host immune system by growing inside eukaryotic cells. A highlight of this session was a talk by William Dietrich on the creative combination of classical mouse genetics and genomics to identify genes involved in susceptibility to *Legionella* infections. A session dealt with the infamous bacterial pathogens *Salmonella* and *Yersinia*, which invade a host and cause serious systemic disease, while another session dealt with another growing threat, opportunistic gram-positive pathogens such as *Staphylococcus, Streptococcus,* and *Bacteriodes*. These pathogens are often resistant to multiple antibiotics and are a common cause of life threatening, hospital-acquired infections, and several talks highlighted new approaches for developing novel antibiotic targets by identifying genes required for virulence in a eukaryotic host. Another session dealt with *Mycobacterium*, the causative agent of tuberculosis. Two sessions focused on the fungal pathogens *Candida*, an opportunistic yeast pathogen that causes serious illness in immunocompromised hosts, and *Histoplasma* and *Coccidiodes* , two fungal pathogens that cause widespread diseases but are less well studied, and in which William Goldman demonstrated how the clever development new molecular genetic tools can facilitate the dissection of the mechanism of pathogenesis in an organism that was previously intractable. One session dealt with how the recent developments in genomics has provided powerful new approaches to study microbial pathogenesis, with highlights being talks by Christopher Gray, on how genome sequences can be used to rapidly determine the structure and function of proteins (proteomics), and Daniel Shoemaker, on the use of high-density arrays (DNA chips) to rapidly identify genes that are turned on or off under specific conditions. Another session dealt with novel model systems for dissecting host-pathogen interactions, and examined the innovative uses of worms, goldfish, and plants to identify virulence genes that also play a role in human pathogenesis.

Finally, Abigail Salyers gave an introspective talk on future challenges facing the field of microbial pathogenesis. Based upon the support and enthusiasm of the participants, the meeting was a resounding success. Thus, a second Microbial Pathogenesis and Host Response meeting will be held in Fall of 1999 participants

## PROGRAM

WEDNESDAY, September 10—7:30 PM

**SESSION 1** TOXIGENIC PATHOGENS: *VIBRIO CHOLERAE* AND TOXINOGENIC *E. COLI*

**Chairperson: R. Taylor,** Dartmouth Medical School, Hanover, New Hampshire

**Keynote Speaker: J. Mekalanos**

THURSDAY, September 11—9:00 AM

**SESSION 2** INTRACELLULAR PATHOGENS: *SHIGELLA*, *LISTERIA*, and *LEGIONELLA*

**Chairperson: P. Sansonetti,** Institut Pasteur, Paris, France

**Keynote Speaker: P. Cossart**

THURSDAY, September 11—2:00 PM

**SESSION 3** POSTER SESSION I: Acute Gram Negative and Intracellular Pathogens

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**Chairpersons:** **V. Miller,** Washington University School of Medicine, St. Louis, Missouri
**J. Slauch,** University of Illinois, Urbana

FRIDAY, September 12—9:00 AM

**SESSION 5** OPPORTUNISTIC GRAM-POSITIVE PATHOGENS: *STAPHYLOCOCCUS*

**Chairperson: M. Schmid,** Microcide Pharmaceutics, Inc.,
Mountain View, California

**Keynote Speaker: A. Tomasz**

FRIDAY, September 12—2:00 PM

**SESSION 6** OPPORTUNISTIC BACTERIAL PATHOGENS II: *STREPTOCOCCUS* AND *BACTERIODES*

**Chairperson: S. Maloy,** University of Illinois, Urbana

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**Chairperson: I. Behlau,** Tufts University School of Medicine,
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**Chairperson: J.-F. Tomb,** The Institute for Genomic Research,
Rockville, Maryland

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**Abigail Salyers**
"Future challenges in microbial pathogenesis"

SUNDAY, September 14—9:00 AM

**SESSION 12** OTHER FUNGAL PATHOGENS: *HISTOPLASMA* AND *COCCIDIODES*

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**Keynote Speaker: W. Goldman**

SUNDAY, September 14—11:15 AM

**SESSION 13** NOVEL MODEL SYSTEMS FOR DISSECTING HOST-PATHOGEN INTERACTIONS

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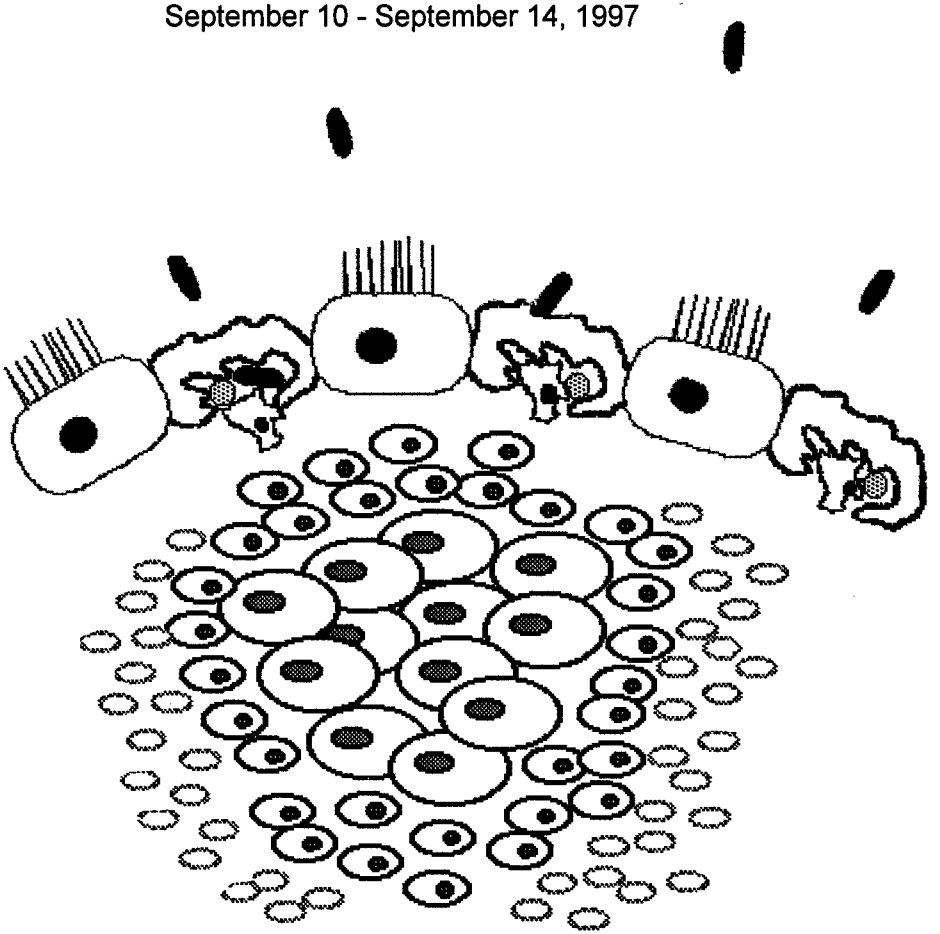
Abstracts of papers presented
at the 1997 meeting on

# MICROBIAL PATHOGENESIS AND HOST RESPONSE

September 10 - September 14, 1997



Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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Arranged by

Stanley Maloy, *University of Illinois*
Ronald K. Taylor, *Dartmouth Medical School*
P.T. Magee, *University of Minnesota*



Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

This meeting is funded in part by the **National Institute of Allergy and Infectious Diseases,** a division of the **National Institutes of Health;** and the **United States Department of the Army.**

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*Cover:* The cartoon on the cover depicts the initial steps of a *Salmonella* infection. *Salmonella* (shown in black) penetrates the intestinal mucosa (shown in red) via M-cells, phagocytic cells involved in antigen processing which are associated with lymphoid follicles called Peyer's patches. In addition to M-cells, the Peyer's patches contain macrophages and lymphocytes (shown in blue), and are surrounded by a network of lymphatic capillaries. The invading bacteria are engulfed by macrophages which are in close contact with the M-cells. Macrophages then carry the bacteria via the lymphatic system to the spleen and liver, where the bacteria replicate. The bacteria subsequently enter the bloodstream causing septicemia. (Modified from Slauch, Taylor, and Maloy. 1997. Survival in a cruel world: how *Vibrio cholerae* and *Salmonella* respond to an unwilling host. Genes & Development 11:1761-1774). We thank Teresa Rebello for help with this figure.

## MICROBIAL PATHOGENESIS AND HOST RESPONSE

Wednesday, Sept. 10 - Sunday, Sept. 14, 1997

| | | |
|---|---|---|
| Wednesday | 7:30 pm | **1** Toxigenic Pathogens: *V. cholerae* and Toxinogenic *E. coli* |
| Thursday | 9:00 am | **2** Intracellular Pathogens: *Shigella, Listeria,* and *Legionella* |
| Thursday | 2:00 pm | **3** Poster Session I |
| Thursday | 4:30 pm | Wine and Cheese Party * |
| Thursday | 7:00 pm | **4** Disseminating Pathogens: *Salmonella* and *Yersinia* |
| Friday | 9:00 am | **5** Opportunistic Gram-Positive Pathogens: *Staphylococcus* |
| Friday | 2:00 pm | **6** Opportunistic Bacterial Pathogens II: *Streptococcus* and *Bacteriodes* |
| Friday | 3:00 pm | **7** Poster Session II |
| Friday | 7:00 pm | **8** Chronic Bacterial Pathogens: *Mycobacterium* |
| Saturday | 9:00 am | **9** Genomics |
| Saturday | 1:00 pm | **10** Opportunistic Yeast Pathogens: *Candida* |
| Saturday | 4:30 pm | **11** Special Perspectives Lecture |
| Saturday | 7:00 pm<br>Following | Banquet<br>Entertainment provided by The Jeff Brown Band |
| Sunday | 9:00 am | **12** Other Fungal Pathogens: *Histoplasma* and *Cocciodiodes* |
| Sunday | 11:15 am | **13** Novel Model Systems for Dissecting Host-Pathogen Interactions |

Poster sessions are located in *Bush Lecture Hall*

* *Airslie Lawn*, weather permitting

Mealtimes at Blackford Hall are as follows:
Breakfast 7:30am-9:00am , Lunch 11:30am-1:30pm, Dinner 5:30pm-7:00pm

Bar times are from 5:00pm until late

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Please note that recording of oral sessions by audio, video or still photography is strictly prohibited except with the advance permission of the author(s), the organizers, and Cold Spring Harbor Laboratory.

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**Overview: R. Taylor**



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**J. Slauch,** University of Illinois, Urbana

**Overview: V. Miller**

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FRIDAY, September 12—3:00 PM

**SESSION 7** POSTER SESSION II: Other Bacterial and Fungal Pathogens

FRIDAY, September 12—7:00 PM

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**BANQUET**

Cockails 6:00 PM Dinner 6:45 PM

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AUTHOR INDEX

# COLONIZATION BY *VIBRIO CHOLERAE*

Ronald K. Taylor
Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03755

The toxin coregulated pilus (TCP) is a type 4 pilus that is required for intestinal colonization by *Vibrio cholerae* strains that cause epidemic cholera. Classical biotype strains of *V. cholerae* elaborate TCP as long bundles of laterally associated pilus fibers that appear morphologically similar to those of bundle forming pilus (BFP) of enteropathogenic *E. coli*. The *tcp* genes that encode the pilin and TCP biogenesis functions are located on a large pathogenicity island termed the TCP-ACF element. In addition to being required for colonization, TCP serves as the receptor for the CTX phage which carries the genes for cholera toxin. Expression of the *tcp* and *ctx* genes is induced in vivo and coordinately regulated as part of the ToxR virulence regulon.

TcpA from *V. cholerae* O1 El Tor biotype or O139 strains shares 82% identity with the classical biotype TcpA. However, unlike classical strains, El Tor and O139 strains express *tcpA* poorly under laboratory culture conditions. Since this has hampered structural and functional studies of El Tor TCP, the *tcpA* gene from El Tor biotype strain C6706 was exchanged into the *tcpA* chromosomal site of classical biotype strain O395. High level expression of the El Tor gene was achieved in this new strain under conditions typical for induction of classical *tcpA*. Interestingly, an atypical autoagglutination phenotype was observed and electron microscopy revealed a preponderance of TCP fibers associated in a criss-cross pattern, unlike the parallel bundles characteristic of classical biotype TCP. Despite this variation in autoagglutination and pattern of pilus fiber association, the classical biotype strains expressing the El Tor biotype TcpA appeared normal for other properties attributable to TCP expression.

Type 4 pilins contain a characteristic carboxy-terminal disulfide loop. Passive immunity provided by monoclonal antibodies raised against TCP, or polyclonal antibodies raised against synthetic peptides, suggests a critical role for this region of the protein in the colonization function of TCP. This role is further supported by the defective colonization properties conferred by site directed mutations within the corresponding region of the *tcpA* gene. Formation of this domain into its correct functional conformation requires the TcpG (DsbA) periplasmic oxidoreductase. Recent determination of the crystal structure of this enzyme provides insight into possible mechanisms of interaction between TcpG and nascent TcpA pilin to mediate conformational maturation of TCP.

# Biochemistry of DNA binding by ToxR, a membrane localized transcription activator in *Vibrio cholerae*

J. Adam Crawford[1] and Victor J. DiRita[1,2]

Department of Microbiology and Immunology[1] and Unit for Laboratory Animal Medicine[2], University of Michigan Medical School, Ann Arbor, Michigan 48109

Expression of several virulence genes in *Vibrio cholerae* is controlled by a transcription activator called ToxR. Among these are the genes encoding cholera toxin (*ctxAB*), the toxin-coregulated pilus (*tcp*), the accessory colonization factor (*acf*), the outer membrane protein OmpU (*ompU*) and aldehyde dehydrogenase (*aldA*). In addition to these, which are positively regulated by ToxR, the *ompT* gene is negatively regulated by ToxR. Some of these genes are indirectly controlled by ToxR, in that they depend on a ToxR-regulated activator called ToxT. Thus, the ToxR regulon is a cascade that can be divided into ToxT-dependent and ToxT-independent branches.

The mechanism of DNA binding and transcription activation by ToxR is not clear. ToxR shares homology at its amino terminus with the carboxy terminal, winged helix-turn-helix domain of OmpR, which controls porin gene expression in *E. coli*. Unlike OmpR, however, a membrane localization domain in ToxR separates the DNA binding domain from a periplasmic domain that is proposed to interact with a membrane protein called ToxS. Membranes of cells expressing ToxR, or ToxR fusion proteins in which the amino terminus is intact, can bind to promoter fragments from the *ctxAB*, *toxT* and *ompU* promoters, and genetic analysis shows that this binding is required for transcription activation.

We developed a DNase I protection assay using membrane fractions as a source of ToxR and ToxS, with the goal of defining the binding requirements for ToxR on the promoters it controls. Three regions of DNA in the *ctxAB* promoter are protected from DNase I digestion; these extend from -122 to -38 and include a previously characterized (Miller *et al.*, Cell *48*, 271-279) series of direct repeats of the sequence TTTTGAT. The *in vitro* footprint of ToxR on the *ctxAB* promoter is consistent with *in vivo* footprinting data from the Taylor lab which identified specific residues required for ToxR binding (Pfau and Taylor, Mol. Microbiol. *20*, 213-222). DNA protected in the *toxT* promoter stretches from -112 to -58 and includes an inverted repeat element between -93 and -58. We previously demonstrated that point mutations in one half-site of this inverted repeat abolish transcription activation by ToxR, while similar mutations in the other half-site do not, thus raising questions about the precise nature and consequences of specific base contacts made by ToxR in this element. More recently, we have begun to analyze the ToxR/DNA interactions at the *ompU* promoter, which has neither inverted nor direct repeat elements but which is bound and activated by ToxR. Preliminary data suggest that ToxR may require sequence dependent secondary structure for binding to this promoter.

The position of ToxR binding with respect to the basal promoter elements in the *toxT*, *ctxAB* and *ompU* promoters is different for all three promoters, making it difficult as yet to conclude which subunits of RNA polymerase may be engaged by ToxR for transcription activation. Based on the data described above, however, as well as genetic data to be presented, a working model for ToxR binding and transcription activation will be presented.

Horizontal Gene Transfer in the Emergence of Vibrio cholerae

John J. Mekalanos
Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Bacterial virulence determinants and antibiotic resistance genes are frequently encoded by accessory genetic elements (bacteriophages, plasmids, transposons, and chromosomal islands). Some of these elements (e.g., self-transmissible plasmids and bacteriophages) have been shown to move horizontally between strains of a given species and sometimes even between different genera. We have been investigating the role of horizontal gene transfer in the evolution of Vibrio cholerae. Specifically, we have been addressing the mechanism of transfer of different segments of DNA encoding antibiotic resistance, novel antigenic structures, or virulence factor production both in vitro and in vivo.

V. cholerae O139 emerged in 1992 to become the first "non-O1" serogroup strain to cause epidemic cholera. Evidence suggests that the emergence of V. cholerae O139 occurred by transfer of novel DNA encoding O139 antigen into an El Tor O1 strain. O139 strains also frequently carry a bacteriophage called kappa. We have characterized the O139 kappa phage and have shown that it encodes a protein (Glo) expressed in lysogenic cells which displays similarity to small eukaryotic G-protein proteins. For example, Glo has at its C- terminus a consensus sequence (CAAX) that determines modification by proteases and isoprenylation enzymes that are typical of eukaryotic cells. We have recently shown that Glo can in fact be modified by yeast prenylation enzymes (B. Akerley, F. Tamanoi, J. Mekalanos, unpublished results). We have shown that Glo is a virulence factor and are currently testing various models for its biological activity. O139 strains can also be distinguished from V. cholerae El Tor O1 by virtue of their characteristic pattern of resistances to antibiotics. We found that these antibiotic resistances are carried on an approximately 62 kb self-transmissible, chromosomally-integrating, element which we have termed the SXT element.

The genes encoding cholera toxin (CT) reside on the CTX genetic element, a 7-9.7 kb segment of DNA present on the chromosome of toxigenic strains but absent in nontoxigenic strains. We have shown that the CTX genetic element corresponds to the genome of a filamentous bacteriophage (designated CTXf). The phage can replicate its DNA in the form of a plasmid or integrate on the chromosome to form a true lysogen. The transcriptional regulator ToxR is known to control the coordinate expression of CT and toxin co-regulated pilus (TCP), an essential intestinal colonization factor of V. cholerae. We have found that the CTXf will not transduce mutants defective in either TCP or ToxR. Thus, a virulence factor (TCP) is the receptor for a bacteriophage encoding another virulence factor (CT) both of which are coordinately regulated by the same virulence regulatory gene (ToxR). Consistent with this model we have observed a high transduction frequency of CTXf from a donor strain to a recipient strain when both strains are co-infected into the gastrointestinal tract of experimental animals.

The genes encoding TCP exist on a large chromosomal "pathogenicity island." Data now suggests that this DNA segment has apparently been recently acquired by V. cholerae through a site-specific recombination reaction mediated by an integrase related to those of coliphages. Thus, the evolutionary steps that convert V. cholerae from a free living aquatic microbe to a human pathogen involve minimally the acquisition of the TCP pathogenicity island (encoding the CTXf receptor and intestinal colonization) and then CTXf (encoding cholera toxin).

Genetic analysis of CTXf has shown that the gene products encoded by the zot and orfU genes are required for phage morphogenesis. Other CTXf-encoded gene products are required for phage DNA replication, regulation and chromosomal site-specific integration. Comparison of the sequence of CTXf genomes from classical and El Tor biotype strains suggest that heteroimmunity exists between different CT converting phages. Thus, emergence of V. cholerae as a pathogen has occurred at least twice based on CTXf type.

INTERACTIONS BETWEEN ENTEROPATHOGENIC *ESCHERICHIA COLI* AND HOST CELLS

Michael S. Donnenberg, Division of Infectious Diseases, University of Maryland at Baltimore, Baltimore, MD 21201

Enteropathogenic *Escherichia coli* (EPEC) may be considered a paradigm for a multistage interaction between pathogen and host cell. Intitial adherence of EPEC to epithelial cells is associated with production of a type IV pilus. All fourteen genes required for synthesis of this pilus in a recombinant *E. coli* host are organized in a single cluster on a large plasmid common to typical EPEC strains. Non-polar mutatgenesis of these genes is beginning to shed light on the biogenesis of these macromolecular structures. EPEC also possess a type III secretion apparatus that is necessary for transducing signals to host cells. This apparatus is encoded by a pathogenicity island. Secretion of three Esp proteins via the type III system is required for activation of a phosphotyrosine-containing receptor that allows EPEC to bind intimately to host cells via the bacterial outer membrane protein intimin. Intimately attached bacteria rest upon a pedestal composed of host cytoskeletal proteins in an arrangement recognized as the attaching and effacing phenotype. The precise molecular interactions that lead to these dramatic alterations in the host cell cytoskeleton remain to be elucidated.

# IMMUNOMODULATION BY THE B-SUBUNITS OF CHOLERA-LIKE ENTEROTOXINS

T. R. Hirst, T. O. Nashar & N. A. Williams

Department of Pathology & Microbiology, University of Bristol, Bristol BS8 1TD, UK.

The cholera-like enterotoxins (including their non-toxic B-subunits) are exceptional immunogens which are able to elicit vigorous mucosal as well as systemic immune responses. They are also able to adjuvant the responses of other antigens; and are reputed to be able to prevent the onset of autoimmunity. Our recent studies have established that these striking immunological properties are explicitly dependent on the B-subunits ability to interact with the eukaryotic cell-surface receptor, GM1-ganglioside. When the immunogenicity of the B-subunit pentamer of *Escherichia coli* heat-labile enterotoxin (EtxB) was compared with that of a receptor-binding mutant EtxB(G33D), the mutant protein failed to induce a mucosal response after oral vaccination and exhibited a much reduced (ca. 200-fold) anti-toxin response when injected systemically [Nashar, et al., (1996) Proc. Natl. Acad. Sci., 93, 223-226]. This can be explained if receptor occupancy triggers either enhanced uptake or presentation or direct modulating effects on lymphocytes. Support for the latter interpretation has come from the finding that the proliferative response of either EtxB- or OVA-primed lymph node cultures in the presence of EtxB is associated with profound alterations in the activation and distribution of lymphocyte subsets. Under such conditions B cells and CD4+ T cells are activated, while CD8+ T cells are depleted by a mechanism involving active induction of apoptosis.

To assess the significance of toxin-receptor interaction in modulating lymphocyte responses we have tested whether EtxB can prevent the onset of collagen-induced arthritis (in mice) when given intra-dermally at the same site as the priming dose of collagen (CII). The results provided dramatic evidence of the potency of EtxB to prevent induction of autoimmune disease. When EtxB was given concurrently with CII it reduced the histological incidence of arthritis (as defined by the presence of any infiltration, hyperplasia or degeneration) from 71% to 0%; it also reduced the mean ankle thickness by 82%. In contrast, EtxB(G33D) had no effect on the onset or severity of disease. Protection was associated with a shift in the Th1/Th2 balance of the anti-CII response as well as a general reduction in the extent of the serological response to CII. These findings demonstrate that EtxB-receptor interaction provides an important mechanism for exerting profound immunomodulatory effects on the immune system and should find widespead applications in the prevention and treatment of both infectious and autoimmune diseases.

PROPER FOLDING OF THE PERIPLASMIC DOMAIN OF CHOLERA TOXIN REGULATOR TOXR IS ENHANCED BY TOXS, AND IS REQUIRED FOR TRANSCRIPTIONAL ACTIVATION BUT NOT DNA BINDING.

James D. Pfau and Ronald K. Taylor
Department of Microbiology
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ToxR and ToxS are integral membrane proteins that activate the transcription of the virulence cascade in *Vibrio cholerae.* Using the P22 challenge phage selection for DNA binding in combination with a genetic screen for transcriptional activation, twelve mutations were isolated in *toxR* and *toxS* (*toxR*$^*$ and *toxS*$^*$ mutations) that produce variant ToxR proteins that bind to the cholera toxin promoter, but fail to activate transcription. In this system, ToxS is required for ToxR mediated transcriptional activation, but not ToxR DNA binding. ToxR can be separated into three different domains: A cytoplasmic N-terminal DNA binding domain, a central transmembrane domain, and a C-terminal periplasmic domain. Seven *toxR*$^*$ mutations included frameshifts and stop codons introduced into periplasmic domain. These had no effect on DNA binding, but appear to be proteolytically processed to a shorter form, suggesting that even short periplasmic deletions perturb the folding of ToxR in the periplasm. Deletion of *toxS* does not appreciably alter the periplasmic stability of ToxR, but ToxS$^*$ variant L33S causes the proteolysis of ToxR suggesting that the natural function of ToxS is to correctly fold the ToxR periplasmic domain. A *toxR*$^*$ mutation in the helix-loop-helix DNA binding domain, E82K, destroys ToxR mediated activation as well. Therefore, alterations that map to the ToxR cytoplasmic DNA binding domain, to the periplasmic domain, or ToxS separate DNA binding activity from activator function. These data support a model where proper folding of the periplasmic domain of ToxR is required for assembly of the activation complex, and is enhanced by ToxS.

REGULATION OF FIMBRIAL EXPRESSION IN ENTERIC BACTERIA. Rob Edwards[1] and Dieter Schifferli[2]. [1]Department of Microbiology, University of Illinois, Urbana, IL 61801; [2]University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA 19104

An early process in the pathogenesis of enteric bacteria is colonization of the intestinal epithelium leading to local multiplication, pathophysiological interactions with the host and further spreading. Attachment is typically mediated by bacterial fimbriae, which are selectively expressed during growth in the intestine. We have analysed the regulation of 987P fimbrial expression of enterotoxigenic *Escherichia coli* (ETEC). Expression of both *fasH*, the transcriptional activator of the 987P fimbrial genes and *fasA*, the major fimbrial subunit are regulated in response to a variety of environmental stimuli. Expression of *fasH* is regulated in response to the carbon status of the growth medium by the cAMP-CRP complex, and in response to both the nitrogen status of the growth medium and the external pH. Expression of *fasA* is activated by FasH, and is also selectively regulated in response to growth temperature by HNS. Regulation of fimbrial expression by carbon and nitrogen gradients is proposed to provide a mechanism allowing preferential colonization of different segments of the intestine by various enteropathogens like ETEC, enteropathogenic *E. coli* and *Vibrio cholerae.*

## THE IMPORTANCE OF FUNCTIONAL BUNDLE-FORMING PILI FOR VIRULENCE IN ENTEROPATHOGENIC *ESCHERICHIA COLI*

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Enteropathogenic *E. coli* (EPEC) infection is hypothesized to proceed by a multi-step mechanism. The first step is a long-range attachment of autoagreggated bacteria to the epithelial cells of the upper small bowel as characteristic, circumscribed microcolonies. Both features of this initial process (autoaggregation and localized adherence of microcolonies) require the elaboration of the bundle-forming pilus (BFP), a member of the Type IV pilus family. Following this primary interaction, the bacteria in the microcolonies intimately adhere to the enterocytes and transmit signals via a classic Type III secretion system that induce the rearrangement of the host cell cytoskeleton. EPEC infection results in effacement of the brushborder microvilli and a presumed reduction in absorptive function of the jejunum leading to a watery diarrhea.

We have shown that expression of normal BFP is necessary and sufficient to produce the *in vitro* phenotypes of autoaggregation (AA) and localized adherence (LA). Since there are no animal models of EPEC infection, we are investigating the role of BFP *in vivo* by studying the effect of defined mutants or wild-type B171-8 on disease in human volunteers. We compared the virulence of null mutants of *bfpA* or *bfpT* (the transcriptional activator of the *bfp* operon, also called *per*) with that of wild type B171-8 at three different doses of inoculum. The expression of BFP was remarkably correlated with diarrheal disease. In contrast, neither mutant was associated with significant diarrhea.

Production of BFP requires the products of the *bfp* operon, which share sequence similarity with proteins known to be involved in Type IV pilus biogenesis in other pathogens but whose precise functions are not yet determined. Two open reading frames in the *bfp* operon, *bfpD* and *bfpF*, both appear to encode nucleotide-binding proteins containing consensus Walker box A sequences, demonstrated in other proteins to be ATP binding sites. Identical amino acid substitutions introduced by site-directed mutagenesis in the invariant Gly-Lys amino acids in the Walker boxes of these proteins have markedly different effects on the BFP. The D-mutant exhibits sparse production of the BFP that form only slender bundles; these D-mutants show neither AA nor LA. The F-mutant, by contrast, exhibits a flamboyant production of the BFP that bundle in a disorganized fashion relative to the wild type; these F-mutants exhibit fairly normal LA, but an exaggerated autoaggregation, forming "autoagglutinates" that are unable to disaggregate at stationary phase, unlike the wild type. The D-mutant is complemented by the expression, in *trans*, of low levels of the wild-type gene; in contrast, the F-mutant is dominant over the wild-type gene in all configurations tested. In-frame deletion mutants of BfpD and BfpF removing at least the Walker box regions of each gene show the same phenotypes as the site-directed point mutants, except that the F deletion mutant is complementable in *trans*. Thus, normal BFP function requires the actions of at least two putative nucleotide binding proteins, the roles of which appear to be distinct and whose mechanisms remain to be elucidated. The BfpF mutants, which produce an abundance of BFP, provide the first available reagents to address the hypothesis that the BFP of EPEC contribute to pathogenesis by a dynamic mechanism rather than serving simply as adhesins. This hypothesis will be tested by extending the human volunteer study described above.

## INTERACTIONS OF *LISTERIA MONOCYTOGENES* WITH MAMMALIAN CELLS : BACTERIAL FACTORS, CELLULAR LIGANDS AND SIGNALLING.

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*Listeria monocytogenes* is a pathogenic bacterium that induces its own uptake into mammalian cells, and spreads from one cell to the other by an actin-based motility process(2).
Entry into host cells involves the bacterial surface proteins InlA(internalin) and InlB. The receptor for InlA is the cell adhesion molecule E-cadherin(5). InlB-mediated entry requires activation of the host protein PI 3-kinase, probably in response to engagement of a receptor(3).
Actin-based movement is mediated by the bacterial surface protein ActA. The N-terminal region of this protein is necessary and sufficient for polymerisation of host cell actin. Recent genetic analysis has revealed a so far unsuspected role for ActA in protecting actin filaments barbed ends from capping proteins(4).
Strategies used by*L. monocytogenes* will be compared to those used by other invasive bacteria(1).

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MOLECULAR BASES OF EPITHELIAL CELL INVASION BY *Shigella flexneri*. Philippe J. Sansonetti, Unité de Pathogénie Microbiennne Moléculaire, INSERM U 389, Institut Pasteur, 28 rue du Docteur Roux, F - 75724 Paris Cédex 15, France.

A major component of the pathogenesis of shigellosis (also called bacillary dysentery) is the capacity of the causative microorganism *Shigella* to invade the epithelial cells that compose the mucosal surface of the colon in humans. The overall invasive process encompasses several steps which can be summarized as follows:

1. Entry of bacteria into epithelial cells. This step appears to involve a complex signalling pathway that elicits a macropinocitic event leading to internalization of the bacterial body. Upon contact with the cell surface, *S. flexneri* activates a Mxi/Spa secretory apparatus encoded by about 25 genes located on a large virulence plasmid of 220 kb. Through this specialized secretory apparatus Ipa invasins are secreted, two of which (IpaB, 62 kDa and IpaC, 42 kDa) form a complex which is itself able to activate entry via its interaction with the host cell membrane as demonstrated by the internalization of beads on which the IpaB-IpaC complex has been adsorbed. Interaction of this molecular complex with the cell surface elicits major rearrangement of the host cell cytoskeleton, essentially polymerization of actin filaments that are all similarly polarized with their positive end oriented towards the inner face of the cytoplasmic membrane. These actin filaments form bundles that support membrane projections which eventually achieve bacterial entry. Active recruitment of the protooncogene pp60$^{c\text{-}src}$ has been demonstrated at the entry site with consequent phosphorylation of cortactin. Also, the role of the small GTPase rho has been demonstrated to be part of the cascade of signals that allows elongation of actin filaments from initial nucleation foci underneath the cell membrane. Work is currently in progress to identify the succession of signals triggered by the invasin complex as well as some of their targets. Another important aspect is injection of bacterial proteins into the target cell via the molecular complex secreted onto its surface. The interaction between IpA and vinculin which remodels the entry focus and makes it fully efficient is an example of such processes.

2. Once intracellular, the bacterium lyses its phagocytic vacuole, escapes into the cytoplasm and starts moving by inducing polar directed polymerization of actin on its surface. This is due to the expression of IcsA, a 120 kDa outer membrane protein, which is only localized at one pole of the microorganism due to cleavage by SopA, a plasmid-encoded surface protease. The host cell ligand(s) that allow(s) actin nucleation-polymerization by interacting with IcsA is not yet known.

3. In the context of polarized epithelial cells, bacteria then reach the intermediate junction and interact with their constitutive components, particularly the cadherins, to form a protrusion which is actively internalized by the adjacent cell. Bacteria then escape the two membranes, reach the cytoplasmic compartment again, and resume actin-driven movement.

This extremely efficient system allows colonization of epithelial monolayers without an extracellular phase for the bacteria.

# LEGIONELLA GENES REQUIRED FOR INTRACELLULAR MULTIPLICATION ACT EARLY IN INFECTION

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*Legionella pneumophila* is a facultative intracellular pathogen that grows equally well within a specialized phagosome in macrophages and on bacterial media. In nature, Legionella *sp.* are found as parasites or endosymbionts of free-living protozoa, suggesting that they evolved specific mechanisms to take advantage of an intracellular lifestyle. We have been testing the hypothesis that specific Legionella genes are required for intracellular growth. Wild-type *L. pneumophila* grows within and kills human macrophages but it is possible to isolate mutants of *L. pneumophila* that are defective for intracellular multiplication and macrophage killing. Transposon mutagenesis generated 55 (out of a total 4564) independent *Legionella* mutants that have a decreased ability to multiply within and kill macrophages. These mutants have been used to identify the wild-type genes required for macrophage killing and intracellular multiplication (*icm* genes). Three non-contiguous regions encoding a total of 23 genes have been identified by sequence and complementation analysis. Only three *icm* genes exhibit homology to known genes in nucleotide and protein databases, these encode a positive regulator of the LysR family (*icmH*) and two proteins involved in plasmid DNA transfer (*icmPO*). The function of the *icm* gene products is related to the ability of the specialized phagosome containing the bacteria to prevent phagosome-lysosome fusion. In contrast to phagosomes containing wild-type bacteria, phagosomes containing *icm* mutant strains fuse with lysosomes within 30 minutes following infection of the macrophages. These results support the hypothesis that *L. pneumophila icm* genes are specifically required to establish and maintain a specialized phagosome inside human macrophages that is conducive for intracellular survival and growth. In addition, high MOI and intimate contact of wild-type Legionella with macrophages causes an immediate cytotoxic effect; *icm* mutants do not produce the cytotoxic effect. These results and the fact that maximal levels of phagosome-lysosome fusion are observed at short times following infection in a variety of *icm* mutants suggests that all of these gene products act at an early step during infection.

*LEGIONELLA PNEUMOPHILA* RESPONDS TO AMINO ACID STARVATION BY EXPRESSING VIRULENCE TRAITS.

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As a parasite of fresh water amoebae and alveolar macrophages, *Legionella pneumophila* must not only replicate in host cells, but also persist as a free-living organism. A link between growth conditions and virulence was identified that can explain how this bacterial pathogen adapts to distinct intracellular and extracellular environments. Results of a series of microbiological and cell biological quantitative assays showed that only post-exponential *L. pneumophila* were cytotoxic, motile, sensitive to sodium, and competent to evade phagosome-lysosome fusion in macrophages, four traits correlated previously with virulence. Likewise, *L. pneumophila* converted between a replicative and a virulent phenotype during growth in macrophages, as judged by immunolocalization of flagella and efficiency of colony formation on medium containing 10 mM sodium chloride. Expression of the virulent phenotype was a response to starvation, since exponential phase cells became cytotoxic, motile, and sodium-sensitive when incubated in broth from stationary-phase cultures, except when it was supplemented with amino acids. Mutant Lp120, identified previously as defective for growth in macrophages, was shown to be defective for expression of all four virulence traits. Thus, the mutation in this strain may identify a component of the regulatory network. Together, these data indicate that while nutrients are plentiful, intracellular *L. pneumophila* are dedicated to replication; when amino acids become limiting, the progeny express virulence factors to escape the spent host, disperse in the environment, and re-establish a protected intracellular niche favorable for growth.

# THE *LEGIONELLA PNEUMOPHILA DOT* VIRULENCE COMPLEX REQUIRED FOR GROWTH IN MACROPHAGES IS CAPABLE OF CONJUGATIVE TRANSFER.

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*Legionella pneumophila* is the causative agent of a form of pneumonia in humans called Legionnaires' disease. This gram negative bacterium survives inside normally bactericidal alveolar macrophages by preventing phagosome-lysosome fusion. Subsequently it forms a novel compartment called a replicative phagosome where it multiplies and eventually lyses the cell. A large number of *Legionella* genes called *dot* (defective for organelle trafficking) have been identified that are required for growth of *Legionella* in macrophages. *Dot* mutants are unable to inhibit phagosome-lysosome fusion and do not replicate in macrophages. The *dot* genes primarily encode for membrane associated proteins which appear to form a large complex. These proteins disrupt the normal endocytic pathway via an undetermined mechanism.

Several pieces of data suggest that the Dot complex resembles a conjugative transfer system. Although the majority of *dot* genes are not similar to known genes, a few of the *dot* genes show a low level of homology to genes involved in bacterial conjugation. DotB has homology to nucleotide-binding proteins including the conjugation gene TrbB of the IncP plasmid RP4. DotG has homology to TrbI of RP4, while DotM and DotL are homologous to TrbA and TrbC of the IncI plasmid R64.

To test whether *Legionella* contains a secretion system resembling a conjugative transfer system, we examined it's ability to transfer the mobilizeable IncQ plasmid RSF1010. Wild type *L. pneumophila* was able to transfer RSF1010 to other *L. pneumophila* strains, as well as to *E. coli*. The transfer was *oriT* dependent, which is consistent with a conjugative mechanism, but not with simple DNA transformation. The transfer was completely dependent on the Dot proteins since specific *dot* mutants were unable to transfer RSF1010.

It is not likely that the *dot* virulence complex is actually transferring DNA into the macrophage since the endocytic pathway is altered very rapidly. It is more likely that the complex is secreting a protein which is inhibiting normal macrophage function. Several examples of related secretion systems in other pathogens include the *virB* operon in *Agrobacterium* and the *ptl* operon in *Bordetella*.

## GENETIC ANALYSIS OF MOUSE SUSCEPTIBILITY TO *Legionella pneumophila*

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Different inbred strains of mice are variably susceptible to some infectious agents, and can be used in genetic studies to identify and study genes involved in the host response to infection. The general approach of our laboratory is to take advantage of genetic polymorphism in inbred mouse strains to study the function of macrophages in host response to pathogens.

*Legionella pneumophila*, the causative agent of Legionnaire's Disease, is a facultative intracellular parasite of macrophages. This intracellular growth is crucial in the pathogenesis of Legionnaire's Disease, since bacterial mutants defective in intracellular growth are avirulent. Interestingly, macrophages from different inbred strains of mouse are either permissive or non-permissive for intracellular *Legionella* replication *in vitro*, and this *in vitro* phenotype reflects susceptibility differences in whole animal *Legionella* infections. We are currently studying 2 mouse strains, called A/J and C57BL/6J, which differ at a single gene, called *Lgn1*, that determines if the macrophages will permit *Legionella* replication.

As part of our work to molecularly clone *Lgn1*, we have genetically mapped this gene to mouse chromosome 13, and have constructed a detailed physical map of genomic clones covering the region where *Lgn1* must reside. Comparison of the mouse *Lgn1* region to the homologous human genomic region on chromosome 5 has revealed extensive homology with a critical region for an inherited human disease called spinal muscular atrophy. Therefore, regional candidate genes can be analyzed for involvement in both disease traits. We are currently testing the candidate genes *Smn* and *Naip* for involvement in the *Legionella* susceptibility phenotype, and are sequencing the mouse genomic region in search of additional candidate genes.

STRUCTURAL AND FUNCTIONAL PROPERTIES OF INVASION PLASMID ANTIGEN C (IpaC) OF *SHIGELLA FLEXNERI.*

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Diarrheal diseases are among the most significant public health problems in the world and *Shigella* spp. are some of the most important agents of infectious diarrhea. The pathogenesis of *Shigella flexneri* is intimately associated with this bacterium's ability to invade and grow in colonic epithelial cells. Invasion of host cells by *S. flexneri* requires active participation by both host and pathogen in a process called "pathogen-induced phagocytosis." The bacterial effectors of entry are invasion plasmid antigens (Ipa) B, C and D which are secreted at the host-pathogen interface *via* the Mxi/Spa translocon. Unfortunately, while the importance of the Ipa proteins in promoting host cell invasion has been clearly demonstrated by molecular genetic analysis, fundamental information on their precise roles in *S. flexneri* pathogenesis and their structure-function relationships is still lacking.

To enhance understanding of the structural and functional features of the Ipa invasins, recombinant IpaB, IpaC and IpaD were prepared and their *in vitro* properties explored. All three proteins associate with cultured Henle 407 cells with IpaD binding being rather weak. Interestingly, IpaC binding to Henle 407 cells results in an altered cell morphology, suggesting IpaC may be a soluble factor capable of influencing the host cell's cytoskeleton. Consistent with this, exogenously added IpaC enhances *S. flexneri* invasion of Henle 407 cells and promotes the uptake of noninvasive bacteria. These data implicate IpaC as a functional component of the *S. flexneri* invasion machinery.

Because protein complexes containing IpaC **and** IpaB have been suggested to be important in the pathogenesis of *S. flexneri*, recombinant IpaC was modified so that its assembly into complexes with other Ipa proteins could be monitored using fluorescence spectroscopy. Interestingly, IpaC could be incorporated into specific complexes with itself or IpaB but not IpaD. These IpaB-IpaC and IpaC-IpaC interactions did not interfere with one another and occurred at nanomolar concentrations of IpaC and IpaB. It will now be possible to investigate relationships between the incorporation of IpaC into homo- and heteropolymers (structure) and its ability to interact with cultured cells and/or their membranes (function).

## THE PORE-FORMING TOXIN AEROLYSIN BINDS TO MICRODOMAINS OF THE PLASMA MEMBRANE AND LEADS TO FRAGMENTATION OF THE ENDOPLASMIC RETICULUM

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Many pathogenic bacteria secrete toxins that are able to form pores in biological membranes. The mechanisms by which these toxins interact with mammalian cells and lead to cell death have only been poorly characterized. We have investigated the effect of the pore-forming toxin aerolysin, produced by *Aeromonas hydrophila*, on Baby Hamster Kidney cells.

We show that the protoxin binds to the cell surface via an 80 kDa GPI-anchored receptor. Interestingly, the toxin does not bind randomly to the plasma membrane but interacts with specific microdomains, the so-called cholesterol-glycolipids "rafts". Our data indicates that the protoxin is then processed to its mature form by host cell proteases. Since the receptor is GPI-anchored and since the toxin is associated "rafts", we propose that clustering in these microdomains effectively promotes processing as well as oligomerization of the toxin, a step that it is a prerequisite for channel formation. We show that channel formation does not lead to disruption of the plasma membrane, since cells exclude dyes such ethidium homodimer-1, but to selective efflux of small ions such as potassium. Morphologically, toxin treatment leads to the appearance of large vacuoles in the cytoplasm of the target cells. Immunofluorescence experiments in which the effect of aerolysin on the distribution of a variety of organelle markers were analysis indicated that the vacuoles originate from early stages of the biosynthetic pathway. This effect was specific since the morphology of other organelles from late stages of the biosynthetic pathway as well as that of organelles along the endocytic pathway was not affected by the toxin. Finally we show that aerolysin inhibits transport of newly synthesized transmembrane proteins to the plasma membrane.

In conclusion, our data indicate that interaction of proaerolysin with a GPI-anchored protein leads to processing of the toxin, oligomerization and channel formation in the plasma membrane as well as to a disorganization of the early biosynthetic pathway, before cell death occurs.

CLONING AND SEQUENCING OF THE *DNAK* AND *GRPE* GENES OF *LEGIONELLA PNEUMOPHILA*

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Heat-shock proteins are synthesized when cells are exposed to thermal and other various environmental stresses. One important family of heat shock proteins is the molecular chaperones, such as DnaK, DnaJ, GrpE, GroEL and GroES. *Legionella pneumophila*, the causative agent of Legionnaires' disease, is a gram negative bacterial pathogen which grows within alveolar macrophages and fresh water protozoa. Pathogenic bacteria like *Legionella pneumophila* may require these chaperones for intracellular survival and multiplication. Although the induction of GroEL and GroES were identified during the infection of macrophages, no identifiable trace of DnaK, DnaJ or GrpE has yet been found in any *Legionella pneumophila* infection. To start studying the possible function of DnaK on *Legionella pneumophila* during the infection of macrophages, we cloned a DNA fragment containing the *dnaK* gene which complements an *Escherichia coli dnaK* ts mutant, HC4102. The length of the DNA fragment was about 4.4 kb. Nucleotide sequence analysis of the region revealed two complete open reading frames encoding both a predicted DnaK protein of 644 aa and a predicted GrpE protein of 199 aa, and also the 5'-end of the predicted *dnaJ* gene organized in the order of *grpE-dnaK-dnaJ*. Consensus heat shock promoter sequences were identified upstream of the start of both *grpE* and *dnaK* transcripts. However, no obvious promoter sequences were detected upstream of *dnaJ*. The transcription start points of *grpE* and *dnaK* were determined by primer extension analysis and the amount of either transcript increased 4-8 fold after heat shock.

ISOLATION OF GENES ESSENTIAL FOR INTRACELLULAR GROWTH OF *LEGIONELLA PNEUMOPHILA*

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*Legionella pneumophila* replicates within a phagosome in cultured cells. The replicative phagosome lacks membrane marker proteins, such as the glycoprotein LAMP-1, that are indicators of the endocytic pathway. To analyze the targeting into this phagosome, we describe the isolation of several genes essential for intracellular growth of *Legionella* using a genetic and cell biological approach. 4900 EMS mutagenized colonies were screened for defects in intracellular growth and trafficking to the replicative phagosome. Of these, 6 mutant strains of *L. pneumophila* were identified which had severe intracellular growth defects in mouse bone marrow derived macrophages. These mutant strains were found to colocalize with LAMP-1. The genes responsible for the defects of three of these strains, *dotH* and *dotN*, were cloned by complementation and found to be located in a region of the chromosome contiguous with several other genes essential for intracellular growth. The predicted DotH protein is 360 amino acids long, and contains an N-terminal secretion signal sequence. DotN consists of 1010 amino acids. Neither DotH nor DotN show homology to previously identified proteins.

A deletion mutation was created in a third gene, *dotI*, which is located in the region adjacent to *dotH*. The *dotI*$^{\Delta}$ strain defective for intracellular growth in macrophages and this defect was complemented by *dotI in trans*. The predicted DotI structure contains two striking features: a single transmembrane domain near the N-terminal end of the protein, and a region of predicted strongly amphipathic β-sheet structure.

# A C-TERMINAL DOMAIN WHICH PARTIALLY OVERLAPS THE SECRETION SIGNAL OF *BORDETELLA PERTUSSIS* ADENYLATE CYCLASE TOXIN IS ESSENTIAL FOR INSERTION OF THE TOXIN INTO TARGET CELL MEMBRANES.

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The adenylate cyclase toxin (CyaA) of *Bordetella pertussis* is a 1706-residue protein which belongs to the RTX (Repeats in ToXin) family of toxins. CyaA is composed of an amino terminal adenylate cyclase (AC) catalytic domain of 400 amino acids linked to a channel-forming and hemolytic domain consisting of 1300-residues. CyaA delivers its AC domain into the cytosol of mammalian cells. Upon activation by host calmodulin, it generates supra-physiological levels of cAMP, impairing host defenses.

The structural gene *hlyA* of the *E. coli* α hemolysin operon, was replaced with *cyaA*, except for the HlyA secretion signal. *hlyC* which is responsible for fatty acid acylation of HlyA was inactivated, and the corresponding gene, *cyaC,* was supplied on a separate plasmid. CyaA was efficiently secreted by the modified *hly* operon and was fully toxic.
CyaAΔ76 lacking the last 76 carboxy-terminal residues was unable to insert into erythrocytes membranes, similarly to a full-length toxin deficient of fatty acid acylation. In contrast, CyaAΔ24 was able to insert into membranes and thus to intoxicate cells. Both CyaAΔ76 and CyaAΔ24 were not secreted by BP. These results indicate that a stretch of 54 amino acids located at the C-terminus of CyaA, and containing a part of the secretion signal, is essential for CyaA insertion into the host membrane, and thus for its toxic activity.

The insertion of CyaAΔ76 into membranes could be fully complemented by a polypeptide encoding the last 216 residues of CyaA ($C_{1490-1706}$), which contains 8 of the 40 $Ca^{2+}$-binding repeats of CyaA. A shorter polypeptide encoding the last 116 residues ($C_{1590-1706}$) and containing the last repeat, which is the least conserved, was unable to complement the toxic activity of CyaAΔ76. The complementation was $Ca^{2+}$-dependent, suggesting that interactions may occur through the repeat region. A specific $Ca^{2+}$-dependent binding between CyaAΔ76 and $C_{1490-1706}$ but not with $C_{1590-1706}$, was demonstrated *in vitro*. This may suggest that the repeat region is needed not only for CyaA interaction with the cell membrane as previously thought, but in fact it may allow binding between toxin monomers that are required for successful penetration of CyaA into cells.

# CHARACTERIZATION OF THE CS17 SUBUNIT GENE FROM ENTEROTOXIGENIC E. COLI AND IDENTIFICATION OF A CLOSELY RELATED COLONIZATION FACTOR

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CS17 is a widespread colonization factor of enterotoxigenic *Escherichia coli*. As part of our effort to identify common ETEC antigens for inclusion in a vaccine, we examined 32 ETEC isolates from a collection obtained from soldiers in the Mid East. Immunoblots of heat, saline extracts of bacteria identified 3 ETEC that expressed proteins that were recognized by antisera specific for CS17. One isolate, DS 37-4 (O114:H21 LT) expressed a protein the size of the prototype CS17 from E20738A. Two isolates, DS 26-1 (O6:H16 LT) and DS 168-1 (O8:H32 LT) expressed proteins that were slightly smaller than the prototype CS17 subunit. The gene expressing the structural subunit of CS17 was cloned from DS37-4 and the DNA sequence was determined. The genes from E20738A, DS26-1, and DS168-1 were obtained on PCR fragments and their DNA sequences were determined. The CS17 genes from prototype strain E20738A and DS37-4 were identical. The CS17 genes from DS26-1 and DS168-1 were identical to each other but distinct from E20738A and DS37-4; the deduced amino acid sequences differed at 17 of 145 residues and there was a deletion of 2 amino acids. These four proteins were more similar to each other than to other members of the CFA/I family: there were 69 differences from the CS17 in DS 37-4 to CFA/I, 59 to CS1, and 76 to CS2. The effect the changes have on virulence, immunity, or adherence is not known. These data raise the question whether the colonization factors from DS26-1 and DS168-1 are variants of CS17 or are new colonization factors, and what criteria should be used to define a colonization factor as unique.

Lipooligosaccharide (LOS) Mutants Of *Haemophilus ducreyi*, Expressing Truncated Oligosaccharide Chains, Exhibit Decreased Adherence and Invasion of Human Keratinocytes In Vitro. A. A. Campagnari[1], M.J. Filiatrault[1], B. Gibson[2] and R. S. Munson, Jr[3]. SUNY at Buffalo[1], Buffalo, New York, University of California at San Francisco[2], San Francisco, California and Ohio State University[3], Columbus, Ohio.

*Haemophilus ducreyi* is a major cause of genital ulcer disease in developing countries and recent sporadic outbreaks of chancroid have occurred in the United States over the past decade. In addition, infection with *H. ducreyi* has been shown to be a risk factor for the heterosexual transmission of the Human Immunodeficiency Virus in Africa and Asia. Despite new research studies involving *H. ducreyi* infection, the events involved in pathogenesis remain obscure. Recent data has shown that *H. ducreyi* adheres to and invades various human cell lines. However the nature of these cell-to-cell interactions, along with the bacterial and eukaryotic components involved, are currently unknown. In this study, we have generated two distinct LOS mutants in *H. ducreyi* strain 35000. The first mutant, designated *H. ducreyi* 1381, was derived by Tn*916* mutagenesis. The second mutant, *H. ducreyi* 35000R, was generated using pyocin selection. The LOS isolated from each of these mutants shows an increased mobility by SDS-PAGE analysis, when compared to the LOS of the wild type strain. Western blot studies, using monoclonal antibodies directed to specific LOS determinants, demonstrated that each mutant lacked the Galβ1-4GlcNAc epitope expressed on the full length, parental LOS molecule. Structural studies have shown that the LOS of *H. ducreyi* 1381 terminates in a glucose residue. Subsequent molecular studies have confirmed that the Tn*916* insertion resulted in the disruption of a gene encoding a heptosyl transferase, which is responsible for adding D-*glycero*-D-*manno*-heptose to the growing oligosaccharide chain. Preliminary structural studies of the LOS of *H. ducreyi* 35000R revealed that this structure is truncated in the heptose core region. The outer membrane protein profiles of *H. ducreyi* 1381, 35000R and the wild type strain 35000 were identical. These three strains were compared in adherence and invasion of human keratinocytes in vitro. In our assay, 21-25% of *H. ducreyi* 35000 cells were adherent to human keratinocytes and 10-12% of these bacteria invaded these cells. In contrast, 5-7% of *H. ducreyi* 1381 and 1-3% of *H. ducreyi* 35000R bound to keratinocytes, while neither strain showed significant invasion (~1%). These data indicate that *H. ducreyi* LOS may be an important bacterial component involved in attachment to and invasion of human keratinocytes.

IDENTIFICATION OF A NOVEL CHROMOSOMALLY-ENCODED TYPE III SECRETION SYSTEM IN YERSINIA ENTEROCOLITICA

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We have identified a region of the chromosome of an O:8 serotype strain of *Yersinia enterocolitica* that contains three genes that are predicted to encode homologs of the type III secretion system identified in a number of other pathogenic microorganisms. This chromosomally-encoded system is distinct from the virulence plasmid-encoded type III secretion system involved in secretion of Yops from the bacterial cell. The first gene identified, named *ysaA* (for *Yersinia* secretion apparatus), codes for a protein 70% similar to the inner membrane transport protein, MxiA, of *Shigella flexneri*. The next gene downstream, *ysaK*, encodes a protein 45% similar to SpaK of *S. flexneri*, a protein with no known function. The final gene, *ysaL*, codes for a protein 67% similar to SpaL of *S. flexneri*, a cytoplasmic protein with ATPase activity. The order of the genes in the newly identified *Y. enterocolitica* chromosomal locus is identical to that found on the *S. flexneri* plasmid. We have constructed an insertion mutation in a plasmid-cured strain of *Y. enterocolitica* by replacement of a 60 bp *Stu*I fragment within the *ysaA* gene with a kanamycin-resistance gene cassette. We have examined the wild type and mutant strain for secretion of proteins in various media. Four proteins of 79, 52, 44, and 27 Kd are secreted by the wild type parent, but not the *ysaA* mutant grown in LB broth with 0.3M NaCl. Introduction of a plasmid containing just the *ysaA* gene is sufficient to restore secretion to the mutant. The mutant is as motile as the wild type parent strain, indicating that this type III secretion system is not involved in flagellar biosynthesis, which is the role of these systems in some bacteria. The *ysaA* mutant is similar to the wild type strain in its ability to enter cultured mammalian cells in vitro. In addition, the mutant is as resistant to serum killing as its parental strain. Thus we have identified a new secretion apparatus in *Y. enterocolitica* that secretes proteins in response to high osmolarity.

DIRECTED MUTAGENESIS OF RECD IN NEISSERIA GONORRHOEAE MS11 INCREASES THE FREQUENCY OF PILIN VARIATION

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*Neisseria gonorrhoeae* can alter the antigenicity of its pili by homologous recombination between incomplete silent pilin loci (*pilS*) and a complete expression locus (*pilE*). Although the outcome of this event resembles gene conversion, the molecular details are poorly understood. In general, gene conversion can result from double chain break (DCB) repair using homologous DNA as the template for repair. In *Eschericia coli*, RecBCD has been shown to be directly involved in the repair of DCBs. RecBCD is a heterotrimeric exonuclease that can convert to a helicase following the transient inactivation of the RecD subunit in vivo. Inactivation of the *recD* gene appears to mimic this conversion to a helicase and is associated with an increase in the frequency of homologous recombination. To assess the role of RecBCD in pilin variation, we inactivated the *recD* gene of *N. gonorrhoeae* MS11 and analyzed the affect of this mutation on pilin variation. Using preliminary data from the *N. gonorrhoeae* FA1090 genome sequencing project, the complete *recD* gene from *N. gonorrhoeae* MS11 was cloned, its nucleotide sequence determined, and the gene inactivated in MS11 by allelic replacement. Southern blotting and PCR confirmed the presence of the mutated allele at the *recD* locus. Inactivation of the *recD* gene in *N. gonorrhoeae* MS11 increased the frequency at which non-parental pilin phenotypes arose on agar plates by approximately 12-fold compared to the isogenic wild-type strain (0.108 +/- 0.020, 0.009 +/- 0.008, respectively) based on the characteristic colony morphology of piliated gonococci. In addition, the nucleotide sequence of the *pilE* gene from the parental MS11*recD* strain and from 20 progeny that displayed a non-parental pilin phenotype was determined. All colonies that evinced a non-parental pilin morphology encoded a non-parental pilin polypeptide. The nucleotide changes in the *pilE* gene of non-parental progeny were similar to changes previously described in wild-type gonococci and attributed to gene conversion. To address the role of DNA transformation in pilin variation, the *recD* gene of MS11*comA*, which is not competent for DNA transformation, was similarly inactivated. Pilin variation in MS11*comArecD* also increased compared to the parental MS11*comA* strain. These preliminary results indicate that pilin variation at *pilE* involves intracellular homologous recombination mediated in part by RecBCD.

THE 987P FIMBRIAL ADHESIN FASG HARBORS SEPARATE BINDING DOMAINS FOR ITS EPITHELIAL GLYCOLIPID AND GLYCOPROTEIN RECEPTORS. Byung-Kwon Choi and Dieter M. Schifferli. University of Pennsylvania School of Veterinary Medicine, Philadelphia, Pennsylvania 19104

The 987P fimbria of enterotoxigenic strains of *Escherichia coli* is a composite structure consisting of one major and two minor subunits, one being the tip adhesin FasG which presents dual binding specificities for sulfatide and a set of glycoprotein receptors on intestinal brush borders. To characterize the interactions of the adhesin with its two types of receptors, mutated and truncated allelic forms of FasG were prepared and used for liposome binding and ligand blotting assays. In view of the acidic nature of sulfatide, twenty single mutants with lysine (K) or arginine (R) to alanine (A) substitutions were generated and used to test whether positively charged residues of FasG are involved in sulfatide-binding. Although *fasG* null mutants are nonfimbriated, only four *fasG* mutants with substitutions demonstrated reduced fimbriation and FasG export. Among the 16 remaining fimbriated mutants, four (K17A, R116A, K118A, R226A) showed reduced binding to sulfatide-containing liposomes and one mutant (K117A) was nonadhesive. This suggested that this lysine, possibly in concert with the other positively-charged residues, participate in a binding pocket interacting with sulfatide by hydrogen bonding and/or salt bridges. These five mutants still bound to the glycoprotein receptor as shown by ligand blotting assays. Truncated FasG proteins fused to the maltose-binding protein were used as inhibitors to show that the C-terminal half of FasG, but not its N-terminal half, is required for binding to the glycoprotein receptor of piglet brush border. This indicated that FasG harbors separate binding domains for its glycolipid and glycoprotein receptors.

**Extracellular secretion of proteins by *Vibrio cholerae*: Are Cholera toxin and chitinase both secreted by the *eps*-encoded pathway?** Terry D. Connell, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, NY 14214.

Extracellular secretion of bioactive molecules is a major virulence determinant for many bacterial pathogens. The severe diarrheal symptoms caused by *V. cholerae* is mediated, in part by the extracellular transport of cholera toxin (CT), an oligomeric heat-labile enterotoxin that binds to receptors on the surface of susceptible eukaryotic cells. A multicomponent secretory apparatus encoded by the *eps* cluster of genes has been demonstrated to be necessary for transport of CT to the extracellular milieu. An extracellular transport signal located on the B polypeptide (CT-B) of the toxin enables the Eps transport apparatus to recognize and secrete the toxin. This transport signal is likely a structural motif, since several other proteins that share little, or no amino acid homology with CT-B are secreted by *V. cholerae*. To identify the extracellular transport signal in a non-CT protein, the chitinase gene (*chi*A) of *V. cholerae* was cloned. Plasmid pTDCC2 was obtained by screening a genomic library of *V. cholerae* 569B for chitinolytic activity using ethylene glycol-chitin agar. Nucleotide sequencing of the cloned DNA fragment in pTDCC2 revealed an ORF of 2,541bp encoding a protein with a predicted molecular mass of 88.7 Kdal. Database searches demonstrated that the ChiA protein shares homology with chitinases expressed by other bacterial species. Secretion of CT by *V. cholerae* requires initial transport of the A and B subunits into the periplasm, a process requiring a signal peptide located at the amino-terminus of the polypeptides. Inspection of the predicted amino acid sequence of ChiA reveals that the protein lacks a similar signal peptide. The data suggest that the initial pathway for secretion of CT and ChiA may differ and that ChiA may not have a periplasmic intermediate. To demonstrate that ChiA is secreted by the *eps*-encoded machinery, a hybrid *chi*A gene was engineered. The polymerase chain reaction was used to incorporate sequences encoded a T7-tag linear epitope into the 3' end of the gene. The hybrid gene was then introduced into *V. cholerae* 569B and into M14 (*eps*E$^-$), an isogenic mutant of 569B deficient for CT secretion. Preliminary results using an anti-T7-tag antibody and Western blotting confirm that ChiA is secreted by the Eps secretion pathway. Investigations are ongoing to identify the transport signal in ChiA.

# EFFECTS OF MUTATIONS IN PTLC AND PTLB ON SECRETION OF PERTUSSIS TOXIN FROM BORDETELLA PERTUSSIS

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Pertussis toxin (PT) is a major virulence factor produced by *Bordetella pertussis*, the etiological agent of whooping cough. Accessory proteins encoded by the *ptl* locus are necessary for the efficient secretion of PT from *B. pertussis*. The *ptl* region maps directly downstream of the co-transcribed *ptx* genes which encode the toxin subunits. The *ptl* locus contains nine open reading frames, *ptlA-I*, the products of which are thought to assemble into a membrane-associated protein transporter. This idea is supported by localization of PtlC-G, and PtlI in membrane fractions prepared from *Bordetella* spp. To characterize the gene products of *ptlC* and *ptlB*, in-frame deletions where made in both genes in the *B. pertussis* strain BP536 which produces and secretes PT, resulting in strains BP536Δ*ptlC1* and BP536Δ*ptlB1*. When assayed for PT secretion, strain BP536Δ*ptlC1* exhibits a 98% reduction in levels of toxin secretion into culture supernatants compared to levels for the parent strain. Constitutive expression of *ptlC* from a broad-host-range vector in strain BP536Δ*ptlC1* restores secretion of PT to wildtype levels. This genetic complementation establishes that *ptlC* is essential for the efficient secretion of PT. Immunoblot analysis of other Ptl proteins in BP536Δ*ptlC1* showed that the steady state levels of PtlI, -E, -F, and G were not affected by the mutation in *ptlC*. Strain BP536Δ*ptlB1* also exhibited a >95% reduction in its ability to secrete pertussis toxin. Genetic complementation of the *ptlB* mutation however did not restore secretion to wildtype levels. In strain BP536Δ*ptlB1*, steady-state levels of PtlE and -F were not affected, however, PtlC, -I and -G were diminished. These results suggest that PtlB may contribute to secretion of PT through the stabilization of other Ptl proteins in the PT transporter.

## CLINICAL TRIALS OF *SHIGELLA FLEXNERI* 2A CANDIDATE VACCINE SC602

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Deletions of the virulence plasmid gene icsA (denoting intra and intercellular spread) and of the chromosomal locus icu (aerobactin) were incorporated into an S. flexneri 2a vaccine designated SC602. Three inpatient clinical trials were conducted using lyophilized SC602 produced under current Good Manufacturing Procedures. All vaccine and challenge inocula were administered concomitantly with sodium bicarbonate. Sequential dose selection studies showed that SC602 caused symptoms of shigellosis when 108 or 106 colony forming units (CFU) were ingested. However, ingestion of 104 CFU was usually followed by asymptomatic excretion of the vaccine strain for over seven days. In a majority of vaccinees, this gradual but persistent intestinal colonization elicited significant numbers of IgA anti-2a LPS secreting lymphocytes in peripheral blood and significant levels of IgA anti-2a LPS in serum and in urine ELISA. In order to evaluate vaccine efficacy, 103 virulent *S. flexneri* 2a were ingested by seven volunteers who had received a 104 CFU dose of SC602 six weeks earlier. Seven unvaccinated volunteers were also challenged. Six of these control volunteers experienced shigellosis with fever and severe diarrhea or dysentery while none of the vaccinees had similar symptoms. However, three vaccinees experienced transient, afebrile diarrhea that did not interfere with normal activities. The latter volunteers had fewer IgA anti-2a ASC and lower serum and urine ELISA titers after vaccination than did the volunteers who experienced no intestinal symptoms. The promising results of these clinic-based studies support planned outpatient studies with SC602 and encourage development of additional *Shigella* vaccine candidates that are attenuated by deletion of the icsA gene.

## THE *SALMONELLA* FLAVOHEMOGLOBIN CONFERS PROTECTION FROM NITROSATIVE STRESS

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Flavohemoglobins are a group of homologous proteins sequenced from an expanding number of prokaryotic and eukaryotic microorganisms. Despite reports of flavohemoglobin knockouts in several species, a defined *in vitro* condition in which this protein is required for normal growth has been elusive. To determine a function for this family of proteins, we have characterized and disrupted the flavohemoglobin (*hmp*) of *Salmonella typhimurium*. *Salmonella typhimurium* harboring a deletion in *hmp* shows normal sensitivity to compounds that cause oxidative stress, but displays increased sensitivity to conditions that produce nitric oxide and to S-nitrosothiol compounds, which cause bacteriostatic nitrosative stress. *LacZ* fusion analyses to the *S. typhimurium hmp* promoter indicate that this protein is induced by nitrogen oxides, but not by S-nitrosothiols. These results suggest a function for the bacterial flavohemoglobins that is independent of oxygen metabolism and uncover a route of protection from nitrosative stress that is distinct from those of other stresses.

# MULTIMERIZATION OF INVASIN PLAYS AN IMPORTANT ROLE IN UPTAKE OF *YERSINIA PSEUDOTUBERCULOSIS* INTO MAMMALIAN CELLS

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The 986 amino acid outer membrane protein invasin of *Yersinia pseudotuberculosis* is the primary bacterially encoded determinant that promotes uptake into mammalian cells through binding to multiple receptors of the $\beta_1$-chain integrin family. Invasin-mediated uptake is regulated by substrate-receptor affinity and by the quantity of invasin ligands and integrin receptors available to participate in this process. Therefore, it has been proposed that bacterial uptake requires high affinity binding of multiple eukaryotic receptors around the surface of the microorganism ("Zipper-Model").

It has been demonstrated that the C-terminal 192 amino acids of invasin are essential for binding to mammalian cells. To determine if additional regions of the invasin protein might contribute or be required for triggering the internalization of bound bacteria, we investigated the ability of latex beads coated with invasin derivatives to enter mammalian cells. The 192 amino acid cell-binding region of invasin was only able to promote cellular penetration at very high concentrations on the surface of the beads. In contrast, beads coated with low concentrations of an invasin derivative comprising the C-terminal 497 amino acids were efficiently internalized by mammalian cells. This indicates the presence of an additional entry-promoting region of invasin required for high efficiency uptake.

One possibility is that this region may play a role in protein-protein interaction. Indeed, gel filtration analysis has shown that invasin is able to form multimers. To further test for invasin multimerization, we took advantage of a genetic system using the DNA-binding domain of phage Lambda repressor as a reporter for protein-protein interactions. The results of this study and the demonstration of a dominant negative effect of cell-binding mutants of invasin on bacterial internalization are consistent with an Inv-Inv interaction.

Furthermore, integrin clustering on the mammalian cell surface can be induced by multimerization of bound invasin molecules. Although local clustering of invasin molecules does not seem to be sufficient to provide the signal for bacterial internalization, antibody inhibition experiments have demonstrated the necessity for multiple sequential invasin-integrin interactions around the microorganism.

# ANTHRAX TOXIN AS A DELIVERY SYSTEM FOR VIRAL AND BACTERIAL T-CELL EPITOPES

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We have been investigating the use of the protective antigen (PA) and lethal factor (LF) components of anthrax toxin as a system for *in vivo* delivery of cytotoxic T-lymphocyte (CTL) epitopes. During intoxication, PA directs the translocation of LF into the cytoplasm of mammalian cells. We have used this system to generate a CTL response in both Balb/c (H-$2^d$) and C57BL/6 (H-$2^b$) mice. CTL epitopes from the nucleoprotein of lymphocytic choriomeningitis virus (LCMV) were fused to the C-terminus of LFn. Balb/c mice were immunized with 30 pmol of the LFn-NP118-126 fusion protein plus PA, and C57BL/6 mice were immunized with 30 pmol of the LFn-NP396-404 fusion plus PA. Two weeks post immunization, spleen cells from the animals were stimulated on irradiated syngeneic spleen cells coated with the cognate synthetic peptide (NP118-126 or NP396-404). Five days later, the stimulated cultures were tested for specific activity by $^{51}Cr$ release assay. In cultures from both haplotypes of mice, specific lysis of the peptide coated cells was significantly higher than lysis of the cells alone. These data suggest that the anthrax toxin system can be used to stimulate an *in vivo* CTL response in at least two different haplotypes of mice. When used to immunize groups of mice the LFn-NP118-126 fusion protein plus PA protected mice against LCMV challenge relative to control mice. We also demonstrate that a single fusion protein can be used to generate a CTL response to two separate pathogens. In these experiments a *Listeria* epitope (LLO91-99) and LCMV NP118-126 are fused in tandem to the C-terminus of LFn and injected with PA into mice. Spleen cells from individual mice showed a CTL response against both the *L. monocytogenes* and LCMV epitopes. We have shown that anthrax toxin is an effective delivery system for epitopes from multiple pathogens, of both viral and bacterial origin, and can prime a CTL response in at least two haplotypes of mice. These data raise the exciting possibility that this technology will be capable of delivering a sufficient number of epitopes to protect against multiple pathogens in a population where many haplotypes are represented.

# INLB AND THE INTERNALIN FAMILY.

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Entry of *L. monocytogenes* into cells is mediated by two genes (1,2):
- *inlA* encodes a 90 kDa protein(InlA or Internalin) used for entry into cells expressing E-cadherin, the receptor for internalin, such as Caco-2 cells.
- *inlB* encodes a 67 kDa protein used for entry into cultured hepatocytes and other cell lines such as HeLa cells, HEp2 cells, and Vero cells.

The structures of InlA (internalin) and InlB are characterized by the presence of leucine rich repeats (LRRs) which are also present in five other *Listeria* proteins.(InlC2, InlC, InlD, InlE, InlF) (3). All Inl proteins except InlC and InlB contain a LPXTG consensus motif which in the case of protein A of *S. aureus* has been shown to anchor the protein into the bacterial cell wall. InlC is totally secreted. InlB is partially secreted and partially present on the bacterial surface (4). We have shown that InlB is associated to the bacterial surface by its 231 amino-acid C-terminal part which is made of 80 amino-acids modules beginning by GW. This region is sufficient to associate heterologous proteins to the bacterial cell wall. Interestingly, surface association also occurs when InlB is added externally to bacteria. This association is productive for invasion as it confers bacterial entry into host cells. GW repeats were also detected, albeit in a larger number in a novel protein called Ami which is undetectable in culture supernatants. Addition of GW repeats to InlB improves anchoring of the protein to the cell surface. "GW" repeats may constitute a novel motif for cell surface anchoring in *Listeria* and have important consequences for the release of surface proteins involved in interactions with eukaryotic cells.

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# IDENTIFICATION OF SALMONELLA PATHOGENICITY ISLAND 2 (SPI2) GENES IN *SALMONELLA CHOLERAESUIS* USING SIGNATURE-TAGGED MUTAGENESIS.

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We have applied signature-tagged mutagenesis (STM) technology to *Salmonella choleraesuis*, a pathogen whose virulence phenotype has not been extensively characterized. Of the initial 95 sequence-tagged mini-Tn*5* mutants screened by intraperitoneal infection of BALB/c mice, five mutants were identified as attenuated out of the pool of cells recovered from infected mouse spleens. Fragments containing the transposon were cloned and the insertion junctions were sequenced.
When screened against the EMBL database, two mutants contained insertions in the *rfa* gene cluster encoding LPS biosynthesis. Two mutants had insertions within the recently described *S. typhimurium* SPI2 pathogenicity island (located at minute 30.7) which encodes a type III secretion system. This gene cluster is distinct from the SP1 *inv/spa* pathogenicity island, which is involved in entry of bacterial cells into the host epithelium, and located at minute 63 in the *S. typhimurium* genome. The location of the transposon insertion in one mutant is in an amino acid biosynthesis gene. Mice and pigs infected with each individually grown mutant resulted in differential degrees of attenuation and protection when compared to the wild-type parent strain or cya crp [cdt] attenuated *S. choleraesuis*. Experiments are currently in progress to further analyze these mutants for use as potential vaccine strains.

REGULATION OF INVASION OF *SALMONELLA TYPHIMURIUM* BY *PHOP* AND IDENTIFICATION OF A NEGATIVE REGULATOR OF *HILA*

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*Salmonella typhimurium* initiates disease via invasion of M cells of ileal Peyers patches. The invasive phenotype of *Salmonella typhimurium* is regulated by environmental conditions such as oxygen, pH, and osmolarity as well as by the *phoP/phoQ* two component regulatory system. We report here the negative regulation of eleven genes required for invasion by *phoP*. Although over expression of invasion genes is observed in a *phoP*- background, a *phoP*- strain does not appear to be hyperinvasive. In a separate study, a search for regulators of *hilA* was conducted. *hilA* has been shown to be an activator of invasion genes. Through transposon mutagenesis of a *hilA*::Tn*5lacZ* strain we have identified a negative regulator of *hilA* which lies upstream of *hilA* in the 40 kb region of *Salmonella* pathogenicity island 1.

ROLE OF ENDOGENOUS CYTOKINES IN INDUCTION OF MURINE CD14 GENE EXPRESSION BY LIPOPOLYSACCHARIDE.

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We previously demonstrated CD14 gene expression in myeloid and epithelial cells of the mouse and showed that its expression in both is modulated by lipopolysaccharide (LPS) but with distinctly different kinetics. Induction in myeloid cells is rapid, peaking 2h after LPS administration, while that in epithelial cells is slower, reaching maximal levels 8-16h after LPS injection. Here we test the hypothesis that the slower induction of CD14 in epithelial cells is an indirect effect of LPS, one mediated by cytokines.

Tumor necrosis factor-$\alpha$ (TNF-$\alpha$) induced a transient increase in plasma levels of CD14 with a peak at 6-8h, and this increase in plasma CD14 antigen was accompanied by increased levels of CD14 mRNA in lung, liver and kidney. *In situ* hybridization studies revealed that CD14 mRNA was induced by TNF-$\alpha$ in both myeloid cells and epithelial cells, the same cells that respond to LPS. Pretreatment of mice with anti-TNF antiserum reduced the LPS-mediated increase in plasma levels of CD14 and significantly reduced the level of induction of CD14 mRNA in selected epithelial cells in the kidney and liver. The antiserum did not appear to block LPS-mediated induction in myeloid cells in the tissues examined. Thus, regulation of CD14 gene expression by LPS differs in epithelial and myeloid cells, with the epithelial response in kidney and liver mediated, in part, by TNF-$\alpha$.

Interleukin-1$\beta$ (IL-1$\beta$), the other major mediator of septic shock, increased CD14 plasma and mRNA levels also, with increased expression in both myeloid and epithelial cells. The effect of pretreatment with anti-IL-1$\beta$ antibodies is currently under investigation.

# A NEW ALLELIC EXCHANGE SYSTEM FOR *PASTEURELLA HAEMOLYTICA* AND CREATION OF A MUTANT STRAIN THAT PRODUCES AND SECRETES ATTENUATED LEUKOTOXIN

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The primary virulence factor of the gram-negative, bovine respiratory pathogen, *Pasteurella haemolytica*, is a secreted, species-specific leukotoxin that is a member of the RTX toxin family. Leukotoxin (LktA) is expressed as a proleukotoxin that is believed to be post-translationally modified by a co-expressed acyltransferase (LktC). We have created a series of shuttle vectors for *P. haemolytica* and have used them to devise an efficient method for targeted chromosomal gene inactivation. Using this system, the *lktC* gene, within the leukotoxin operon, was insertionally inactivated using a nonpolar, promoterless chloramphenicol resistance cassette. The insertion in *lktC* did not affect expression of the downstream *lktABD* genes and created an operon fusion to the leukotoxin promoter. The mutant strain secreted a proleukotoxin that was neither leukotoxic nor hemolytic. By Western blot analysis, the proleukotoxin was recognized by polyclonal bovine convalescent serum and by both neutralizing and non-neutralizing monoclonal antibodies raised against the leukotoxin. Expression of the *lktC* gene *in trans* restored cyotoxicity, confirming that LktC is required for activation of the proleukotoxin to the mature leukotoxin. Experiments have been initiated to examine the virulence and vaccine potential of the mutant strain.

BEHAVIOUR OF *SHIGELLA FLEXNERI IPAH* MUTANTS IN MOUSE J774 CELLS AND IN HUMAN MONOCYTE-DERIVED MACROPHAGES.

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Human monocyte-derived macrophages (HMDM) infected *in vitro* with virulent *Shigella flexneri* 5, strain M9OT-W, undergo cell death by a rapid cytolytic process that resembles oncosis, while a similar infection of the mouse macrophage-cell line, J774, results in macrophage cell death by apoptosis. In both types of macrophages, IL-1β is released into the culture supernatants, indicating loss of plasma membrane integrity. *S. flexneri ipa*B mutants fail to kill either type of macrophages. Isogenic avirulent strain M9OT-55 remained within endocytic vacuoles, from where it was recovered as CFU 2-3 logs higher than the virulent strains. Infection of HMDM and J774 cells with *S. flexneri ipa*H mutants further illustrates the difference between these two types of macrophages. *ipa*H mutants behave like wild-type strains in HeLa cell invasion assays while giving a greatly exaggerated inflammatory response, as compared to wild-type strains, in the guinea pig Sereny reaction. In HMDM, IpaH mutants behaved similar to the wild-type strain. Very few organisms were recovered after incubation and the macrophages were lysed by a process distinct from apoptosis. In contrast, infection of J774 with strains containing a deletion in the *ipa*H$_{7.8}$ gene resulted in CFU recovery that were 2-3 logs higher than the wild-type virulent strain and approached values exceeding those seen with an avirulent strain. However, due to the presence of IpaB, the macrophages were seen to undergo apoptosis releasing IL-1β as seen with the wild type strain. It took longer for *ipa*H$_{7.8}$ mutant-infected J774 cells to undergo cell death with releas e of enzymes and cytokines as assayed by light microscopy, lactate dehydrogenase (LDH) release assays, chloroquine experiments and flow cytometric analysis. Strains with deletions either in *ipa*H$_{4.5}$ or *ipa*H$_{9.8}$ gave CFU that were comparable to the wild-type strain. Recovery of bacteria after infection with double or triple *ipa*H mutants were similar to that seen with *ipa*H$_{7.8}$ mutant alone. The behaviour of *ipa*H$_{7.8}$ mutants in J774 cells indicate that one function of the *ipa*H$_{7.8}$ gene may be to facilitate the rate of escape of the virulent bacteria from the macrophage endocytic vacuole into the cytoplasm.

# THE *tia* GENE OF THE PROTOTYPICAL ETEC STRAIN H10407 IS ENCODED ON A LARGE CHROMOSOMAL ELEMENT INSERTED WITHIN THE *selC* tRNA GENE.

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The *tia* gene encodes a 25 kDa outer membrane protein, and directs enterotoxigenic *E. coli* to adhere to and invade epithelial cell lines of gastrointestinal origin. *tia* was originally cloned from ETEC strain H10407 on two cosmids, pET101 and pET102. These two cosmids span approximately 48.5 kb of the ETEC chromosome and overlap by approximately 26.7 kb. DNA sequence analysis of the flanking ends of the inserts of pET101 and pET102 demonstrated the presence of a 16 bp direct repeat (DR) element. The sequence of the DR (5'-TTCGACTCCTGTGATC-3') is identical to that of the DR at ends of the *E. Coli* pathogenicity islands PAI-1 from the uropathogenic strain 536, and the LEE from enteropathogenic *E. coli*. In ETEC H10407 these DRs flank a chromosomal insertion of approximately 46 kb within the selC gene at the same location as PAI-1 and the LEE. The G+C content of this large insertion is approximately 43.7 % (based on 37 kb of sequence data), vs 51 % for the surrounding *E. coli* chromosome. Analysis of DNA sequence upstream from *tia* revealed the presence of a cryptic integrase gene with approximately 70 % identity to the prophage P4 integrase. Downstream from *tia* resides a 1.3 kb element with 94 % identity to the nucleotide sequence of IS2 (*E. coli*). Further downstream from the IS2-like region lies a candidate 1.2 kb open reading frame encoding a predicted product with 69 % identity to the integrase of retronphage phi-R73. Together, these data demonstrate the presence of a large chromosomal element within the H10407 genome which shares multiple characteristics conserved among defined pathogenicity islands. Our data raise the possibility that additional virulence factors are encoded in this region.

# CHARACTERIZATION OF A POTENTIAL PATHOGENICITY ISLAND OF ENTEROTOXIGENIC *BACTEROIDES FRAGILIS* (ETBF) STRAINS

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Enterotoxigenic *B. fragilis* (ETBF) strains which produce a 20 kDa zinc metalloprotease toxin (BFT) have been associated with diarrheal disease of animals and young children. We have previously reported the cloning and sequencing of the entire *B. fragilis* toxin gene (*bft*) from strain 86-5443-2-2 (piglet isolate) (Infect Immun., 1997. 65:1007-1013). Using *bft* as a probe, the gene was identified in 74/77 human and animals ETBF strains but only 2/97 non-toxigenic *B. fragilis* (NTBF) strains. The region flanking *bft* was mapped with several restriction enzymes and 8 restriction fragments adjacent to *bft* were used to probe colony blots of 77 ETBF and 97 NTBF strains. All 74 *bft*- positive ETBF strains hybridized to the 8 probes spanning a *ca.* 18 kb chromosomal region; however, this 18 kb region was absent in the 3 ETBF strains lacking *bft*. None of the 97 NTBF strains possessed a *ca.* 6 kb region containing *bft*, and 47 of the 97 (48%) NTBF strains lacked the entire 18 kb region. Of note, the 2 NTBF strains containing *bft* did not have a *ca.* 12 kb region upstream of *bft*. A *ca.* 9 kb fragment flanking the *bft* gene has been sequenced. Analysis of this data revealed several open reading frames (ORF) of which 3 are of particular interest (ORFs 1, 2 and 3). ORF 1 and ORF3 encode proteins with significant homology to mobilization proteins, and ORF2 encodes a protein with significant homology to metalloprotease proteins, but only 50% similarity and 30% identity to BFT. These results suggest: 1) the *bft* genes are flanked by at least 18 kb of DNA largely unique to ETBF strains indicating a putative pathogenicity island (BfPAI), 2) another metalloprotease protein (distinct from BFT) present in ETBF strains may contribute to the pathogenicity and variable virulence of these diarrheagenic strains, and 3) the BfPAI may be mobilized among different *Bacteroides* strains, and possibly among different species of intestinal bacteria.

# CHARACTERIZATION OF LISTERIA MONOCYTOGENES INTRACELLULAR GENE EXPRESSION USING THE FLUORESCENT GREEN PROTEIN OF AEQUOREA VICTORIA

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*Listeria monocytogenes* is a gram-positive facultative intracellular parasite of mammalian cells. Several gene products have been identified that are necessary for invasion, intracellular growth, and cell-to-cell spread of *L. monocytogenes*, and each of these gene products is regulated by a transcriptional activator known as PrfA. PrfA appears to temporally regulate *L. monocytogenes* virulence expression such that specific gene products are only expressed within specific host cell compartments. We have used the green fluorescent protein (GFP) gene reporter system of *Aequorea victoria* to measure the expression of *L. monocytogenes* virulence genes within host cells by fluorescent microscopy. *gfp* fusions to *actA*, whose gene product is required for *L. monocytogenes* actin-based motility and cell-to-cell spread, have indicated that *actA* is expressed at low to undetectable amounts in bacteria grown in vitro or in bacteria located within host cell vacuoles. Once *L. monocytogenes* escapes from host vacuoles into the cytosol, *actA* expression increases (based on observed fluorescence) and continues at high levels as the bacteria spread to adjacent cells. Our studies indicate that the GFP reporter system can be used to distinguish intracellular bacterial gene expression within specific host cell compartments, and to define temporal virulence gene regulation.

POTENTIAL EVOLUTION OF AN INTRACELLULAR BACTERIUM FROM A PROTOZOAN PARASITE INTO A LEGIONNAIRES' DISEASE CAUSING AGENT. **Lian-Yong Gao, Omar S. Harb, Barbara J. Stone, and Yousef Abu Kwaik.** University of Kentucky Medical Center, Lexington, KY 40536

To test the hypothesis that *L. pneumophila* utilizes the same mechanisms to parasitize both mammalian and protozoan cells, we screened a bank of 5280 miniTn*10*::kan transposon insertion mutants of *L. pneumophila* for potential mutants that exhibited different phenotypes of cytotoxicity and intracellular replication within U937 macrophages and *Acanthamoeba polyphaga*. In addition to 89 mutants that were defective in both host cells, we identified another 32 mutants with varying degrees of defects in cytotoxicity, intracellular survival, and replication within macrophages but exhibited wild type phenotypes within protozoa, and these were designated as *mil* (macrophage infectivity loci). Transmission electron microscopy showed that 2 of the *mil* mutants recruited the rough endoplasmic reticulum (RER) and the mitochondria around the phagosome within *A. polyphaga*, but failed to recruit the RER in macrophages. Our data showed that *L. pneumophila* utilizes unique mechanisms to parasitize mammalian cells. We speculate that during ecological co-evolution of *L. pneumophila* as a protozoan parasite the bacterium acquired loci for survival within macrophages or loci for multiple redundant mechanisms to parasitize protozoa, and some of these redundant mechanisms do not function within macrophages.

MECHANISM OF INTERACTION WITH EPITHELIAL CELLS OF UROPATHOGENIC AND DIARRHEA-ASSOCIATED *ESCHERICHIA COLI* STRAINS EXPRESSING THE " AFA" ADHESION SYSTEM.

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Urinary tract infections (UTI), of which *Escherichia coli* is the major causative agent are among the most common human infections. Additionally, diarrheagenic *E. coli* strains continue to present a major health problem in many areas of the world. Among the pathogenicity factors of the *E. coli* isolates, the afimbrial adhesive structure (AFA) encoded by the *afa* gene clusters is the only one that is produced by strains associated with both UTI and diarrhea. We demonstrated that the AFA adhesive structure is composed of the AfaD and AfaE proteins that play distinct roles during bacterial-epithelial cell interactions. The AfaE adhesin is essential for the initial adherence of bacteria to epithelial cells while the AfaD product is necessary for entry of adherent bacteria into the cells. These observations arise from experiments using human undifferentiated HeLa cells, as well as human differentiated cell lines such as intestinal Caco-2 cells or urothelial UROtsa cells. Prolonged incubation of adherent bacteria with HeLa cells is accompanied by an increase of the internalisation process and appearance of damages in the epithelial cells. This two-step mechanism of interaction of *afa* strains with epithelial cells could explain the clinical and epidemiological data that associate these pathogenic *E. coli* with recurrent UTI as well as persistent diarrhea.

ENTEROPATHOGENIC *ESCHERICHIA COLI* (EPEC)-MEDIATED ANTIPHAGOCYTOSIS

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EPEC is a leading cause of infantile diarrhea, killing up to one million children per year worldwide. It is well established that EPEC interacts with intestinal epithelial cells, activating host signaling pathways, leading to cytoskeletal rearrangements and ultimately disease. Recently, tyrosine dephosphorylation of several host proteins has been observed in cultured epithelial cells during EPEC infection, but its role in pathogenesis remained unknown. An invasive pathogen, *Yersinia*, induces tyrosine dephosphorylation of host proteins to avoid phagocytosis by macrophages. Although EPEC is a non-invasive pathogen, it is possible that these tyrosine dephosphorylation events play a role in inhibiting uptake by intestinal phagocytic cells. Using the murine macrophage-like cell line J774A.1, we found that EPEC could inhibit its own uptake by macrophages. Additionally, EPEC signaling inhibited the uptake of an invasive pathogen, *Salmonella typhimurium*, by macrophages, suggesting that EPEC-mediated antiphagocytosis was affecting a general uptake mechanism. The EPEC antiphagocytic phenotype was dependent upon the type III pathway-mediated secretion of EPEC proteins. Furthermore, tyrosine dephosphorylation of high molecular weight host proteins was observed following infection with secretion competent EPEC, but not with secretion deficient mutants. Inhibition of protein tyrosine phosphatases by pervanadate treatment increased the number of intracellular EPEC to levels seen with secretion deficient mutants, suggesting that the dephosphorylation events were linked to antiphagocytosis. Taken together, the present findings suggest a role for EPEC-mediated antiphagocytosis during the EPEC infection process.

## CONSTRUCTION, CHARACTERIZATION, AND ANALYSIS OF CHIMERIC AND DELETION MUTANT OPA PROTEINS IN *NEISSERIA GONORRHOEAE*

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*Neisseria gonorrhoeae* MS11 contains 11 genes encoding opacity proteins (Opa), a family of outer membrane proteins, which contribute to colony opacity and are believed to be involved in a number of functions including interaction with the host cells. Opa proteins are very similar to each other with the exception of one semi-variable (SV) and two hypervariable (HV) regions. Particular Opa proteins confer on *N. gonorrhoeae* the ability to adhere to tissue culture cells of human or non-human origin and to be taken up by human conjunctiva (Chang). Other Opa proteins appear to be involved in the interaction of *N. gonorrhoeae* with human polymorphonuclear leukocytes (PMN's).

In order to determine functional domains of the Opa proteins of *N. gonorrhoeae,* chimeric *opa*A, B, and C genes of *N. gonorrhoeae* MS11 were constructed. *opa*A, B, and C were selected from the repertoire of strain MS11 for the following reasons: OpaA mediates the adherence and uptake of *N. gonorrhoeae* by heparan sulfate-expressing epithelial cells, but this Opa renders *N. gonorrhoeae* non-reactive with PMN's *in vitro* ; OpaC expressing *N. gonorrhoeae* adhere to both Chang cells and PMN's but are only internalized by PMN's and not Chang cells; OpaB expressing *N. gonorrhoeae* do not interact with Chang cells but do adhere to and are internalized by PMN's, thereby resembling *N. gonorrhoeae* that express the other Opa proteins (D, E, F, etc.) of this strain's repertoire. That portion of each *opa* gene that encodes its $HV_2$ hypervariable region was replaced by the analogous region of the other two *opa* genes. In this way, six chimeric recombinant *opa* genes were constructed ($AB_2$, $AC_2$, $BA_2$, $BC_2$, $CA_2$, and $CB_2$). Mutants of *opa*A were also constructed such that the $HV_1$ (ÆHV$_1$) and $HV_2$ (ÆHV$_2$) regions of OpaA were deleted. Chimeric and deletion *opa* genes were expressed from a plasmid under control of a synthetic*opa* promoter in a strain of *N. gonorrhoeae* MS11 in which the *opa*A gene had been mutated by insertion of a chloramphenicol resistance marker.

*N. gonorrhoeae* MS11 expressing chimeric and deletion *opa* genes were examined in several assays for Opa structure-function correlations. These include binding assays with heparin and the ectodomain of Chang cell surface heparan sulfate glycoproteins, adherence to, and internalization by tissue culture cells, susceptibility to polysulfated compounds, growth and colony characteristics and influence on whole cell (gonoccocal) electrophoretic mobility.

# ROLE OF LIPOPOLYSACCHARIDE IN BINDING AND INTERNALIZATION OF *CAMPYLOBACTER JEJUNI* TO CULTURED EPITHELIAL CELLS

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A *Campylobacter jejuni* gene encoding a functional homolog of the *Salmonella typhimurium rfaC* gene was isolated and characterized. This *C. jejuni* gene, designated *waaC*, complemented a heptose-deficient strain of *S. typhimurium* as judged by novobiocin sensitivity, lipopolysaccharide (LPS) specific-phage sensitivity, and LPS gel profiles. Sequence analysis of the cloned *C. jejuni* DNA insert revealed an open reading frame (ORF) of 1029 nucleotides that was capable of encoding a protein of 342 amino acids with a calculated molecular mass of 39,316 Da. Translation of this gene in a cell-free system yielded a protein with a $M_r$ of 39,000 as judged by SDS-PAGE. The deduced amino acid sequence of the *C. jejuni* WaaC protein exhibited similarity with the products of the *rfaC* genes from other Gram-negative bacteria. An isogenic *C. jejuni* WaaC mutant was generated via homologous recombination with a suicide vector containing an internal fragment of the *waaC* gene. Polyacrylamide gel electrophoresis of LPS extracts using the tricine buffering system revealed that the LPS of the *C. jejuni* WaaC mutant had a greater mobility than that of the parent. *In vitro* binding and internalization assays revealed that the internalization of the WaaC mutant was significantly reduced when compared to the parental isolate. Outer membrane protein extracts of the mutant lacked a 62 kDa band, which was determined to be flagellin, as judged by polyacrylamide gel electrophoresis coupled with immunoblot analysis with an anti-flagellin specific antibody. These data suggest that the reduction in the internalization of the WaaC mutant may be due to the non-assembly of flagella.

*NEISSERIA GONORRHOEAE* OPA-DEPENDENT INTERACTIONS WITH CD66 CARCINOEMBRYONIC ANTIGEN RECEPTORS INFLUENCES TISSUE RESPONSE AND TRIGGERS THE STIMULATION OF A SRC-FAMILY TYROSINE KINASE AND RAC1-DEPENDENT SIGNALLING PATHWAY.

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Among the eleven variable opacity (Opa) proteins encoded by N. gonorrhoeae MS11, one Opa mediates binding to heparan sulfate proteoglycan receptors, while the other ten Opa's possess various binding specificities for at least four of the CD66 carcinoembryonic antigens which are expressed on epithelia, endothelia and phagocytic cells. The biological significance of these specificity patterns is illustrated by a correlation between the competence of individual Opas to interact with one of these proteins (CD66a) and the level of gonococcal binding to primary endothelial cells after stimulation with the inflammatory cytokine TNFα. In addition, this same Opa-specificity is also observed for the ability of both *N. gonorrhoeae* and *E. coli* strains expressing defined recombinant Opa proteins to induce a strong oxidative burst in polymorphonuclear neutrophils.

Gonococcal adherence to transfected HeLa cells expressing individual CD66 receptors results in bacterial internalisation. Interestingly, expression of a natural CD66d splice variant which lacks the full length cytoplasmic domain of this receptor allows bacterial adherence but no bacterial uptake. This suggests that an active receptor-mediated phagocytosis process occurs which is dependent upon sequences which are presented only by the full length receptor. Consistent with this model, we have used both the in vitro-differentiated myelomonocytic JOSK-M cell line and primary neutrophils to show that recombinant $Opa_{52}$-expressing gonococci or *E. coli* activate a signalling cascade which involves Src-like protein tyrosine kinases, Rac1, PAK and the Jun-N-terminal kinase (JNK). The induced signalling events are mimicked by CD66-specific $F(ab)_2$ fragments, and are Opa-dependent since piliated, Opa-negative gonococci, non-pathogenic *Neisseria cinerea* and *E. coli* do not stimulate a similar response. Inhibition of Src-like kinases or of Rac1 prevents the uptake of $Opa_{52}$-expressing bacteria, demonstrating the crucial role of this signal cascade for the opsonin-independent, CD66-mediated phagocytosis of pathogenic gonococci.

**Tn*552* transposition *in vitro*: A tool for rapid, efficient and random insertion mutagenesis**

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A simple, efficient procedure for transposition of Tn*552 in vitro* has been developed. This system is useful for random insertion of a DNA element into a target of choice. Essentially any piece of DNA could be cloned between the transposon ends; *i.e.* antibiotic resistance genes, primer binding sites to be used for DNA sequencing or reporter gene systems to study expression and/or regulation. Such insertions could then be screened either in *E. coli* or directly in the host organism.

Tn*552* transposase has been shown to catalyze concerted transposition events *in vitro*. The reaction requires a substrate transposon molecule, a target molecule and a single protein, Tn*552* transposase (TnpA). The Tn*552* substrate requires that only 48 base pairs of the transposon ends be present to allow transposition. TnpA, containing a six His tag, is readily purified in soluble form. Reaction conditions have been optimized so that >95% of the products are concerted events.

In our reactions, we typically recover transposition events into 1% of pUC19 plasmid molecules or into 10% of cosmid DNA molecules present in the reaction. For example, in a standard reaction using 50-100 ng of target cosmid DNA, we routinely recover several thousand of independent insertions following electroporation into *E. coli*. Under the conditions tested, the sites of insertion are, for all intents and purposes, random; insertion sites are seen scattered throughout a plasmid or cosmid by restriction analysis. Furthermore, we have shown that the Tn*552* substrate can insert at essentially every base pair, with little preference for target site.

# LIPOOLIGOSACCHARIDE BASED CONJUGATES AFFORD PROTECTION AGAINST OTITIS MEDIA CAUSED BY NONTYPEABLE HAEMOPHILUS INFLUENZAE IN CHINCHILLAS

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Detoxified lipooligosaccharide (dLOS)-protein conjugates from nontypeable Haemophilus influenzae (NTHi) elicited a significant rise of anti-LOS antibodies with bactericidal activity in rabbits (Gu, et al. Infect. Immun. 64:4047-53). In this study, we evaluated whether vaccination with these conjugates would protect against NTHi otitis media in a chinchilla model. Fifty-eight chinchillas received three subcutaneous or intramuscular injections of 25 ug of dLOS-TT (tetanus toxoid), dLOS-HMP (high molecular weight proteins from NTHi) or saline (control) in Freund's adjuvant four weeks apart. Two weeks after the last injection, the chinchillas were challenged by intrabullar inoculation with 140 CFU of NTHi strain 9274 in the right middle ear. Otitis media with culture-positive NTHi effusions developed in all controls (19/19) and 56% of conjugate-immunized animals (22/39) during a period of 21 days ($p < 0.001$). The incidence of infection in the unchallenged ear was reduced 51% in the conjugate groups (10/39) as compared with the controls (10/19) ($p < 0.05$). The incidence of inner ear infection was also reduced 51% in the conjugate groups (11/39) as compared with the controls (11/19) ($p < 0.05$). Bacterial counts of the middle ear effusions on 3, 7, 14 and 21 days postchallenge were significantly lower in the conjugate groups than in the controls ($p < 0.01$). All vaccinated animals responded with elevated serum titers of anti-LOS of which 49% (19/39) demonstrated complement-mediated bactericidal activity against the homologous strain. There was no advantage to dLOS-HMP when compared to dLOS-TT. These data indicated that active immunization with LOS based conjugates reduce the incidence of NTHi-induced otitis media.

# EXPRESSION OF RHESUS ROTAVIRUS VP4 IN A VACCINE STRAIN OF SHIGELLA FLEXNERI

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Temperate bacteriophages have developed successful strategies to integrate their DNA into their host chromosome and survive like a part of the host genome. Many of these site-specific recombination systems have been well characterized, however, use of these genetic elements to integrate and express foreign genes from other infectious agent has not been utilized. Here we present the successful integration of full-length non-fused Rhesus rotavirus (RRV) outer membrane spike forming protein VP4 gene into a Shigella flexneri strain using a site-specific recombination system of Shigella flexneri bacteriophage V (SfV). RRV VP4 cDNA was cloned under the control of a Ptac promoter in a suicide vector which contains the integration gene (int) and the attachment site (attP) of SfV. This plasmid was electroporated into a vaccine strain of S. flexneri. Southern hybridization revealed that the VP4 gene and the suicide vector had been inserted into the expected unique site located on the chromosome of S. flexneri strain. Western blot analysis showed that the inserted VP4 gene was stably expressed. The recommbinant shigella strain expressing rotavirus VP4 is currently being used as an immunogen to study specific immune responses against rotavirus in an animal model.

SEQUENCE AND MAPPING OF *SHIGELLA FLEXNERI* 5 *ipaH*$_{9.8}$ AND EFFECT ON SEVERITY OF KERATOCONJUNCTIVITIS IN GUINEA PIGS PRODUCED BY *ipaH* DELETION MUTANTS.

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The *ipaH* genes comprise a multicopy gene family located on both the invasion plasmid and chromosome of *Shigella* species and EIEC. Convalescent sera from primate hosts and guinea pigs infected with *Shigella* recognize the product of the *ipaH* gene. Previously, four of the five *ipaH* genes present on the invasion plasmid of *S. flexneri* 5 (pWR100) have been sequenced and shown to contain a conserved carboxy terminal region of 600 bp. Two of these, *ipaH*$_{7.8}$ and *ipaH*$_{4.5}$, are located 8 kb upstream of the *ipaBCDARJ* genes and encode proteins of 60 kDA and 65 kDa, while *ipaH*$_{2.5}$ and *ipaH*$_{1.4}$ appear to be truncated at their amino terminal end. The amino terminal half of IpaH$_{4.5}$ and IpaH$_{7.8}$ contains leucine-rich internal repeat structures similar to those found in other members of the leucine-rich glycoprotein (LRG) family. The *ipaH*$_{9.8}$ gene has now been sequenced and encodes a 62 kDa protein with a structure similar to that of IpaH$_{7.8}$ and IpaH$_{4.5}$, i.e. it contains a unique leucine-rich repeat motif at its amino terminal end. The *ipaH*$_{9.8}$ gene is located on pWR100 in the region between *virG* (*icsA*) and *virK* while *ipa*$_{2.5}$ has been mapped 5.5 kb upstream of the *virF* gene. The *ipaH*$_{9.8}$ gene is flanked on the 5' end by sequences homologous to those found in the regions of plasmid mini-F that are involved in partitioning and on the 3' end by sequences homologous to plasmid *ssi* sequences. A complete *IS1* sequence is found about 1650 bp from the 3' stop codon of the *ipaH*$_{9.8}$ gene in *S. flexneri* 5. Single, double and triple *ipaH* mutants in *S. flexneri* 2a have been constructed via suicide vectors. These mutants do not affect HeLa cell invasion in tissue culture. The mutants were then tested at two different concentrations in the guinea pig keratoconjunctivitis model (Sereny test). Results indicate that *ipaH* deletion mutant strains produced a more severe infection with much greater inflammatory response and purulence than was produced by the parent 2a strain. Thus *ipaH* may play a role in modulating host response to infection.

## ROLE OF INVASION IN *PSEUDOMONAS AERUGINOSA*-MEDIATED EPITHELIAL CELL DAMAGE

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*Pseudomonas* aeruginosa is one of the most virulent opportunistic pathogens of man, causing nosocomial pneumonia, bacteremia, and urinary tract infections in immunocompromised hosts. The morbidity of *P. aeruginosa* infections results from the ability of the bacterium to colonize previously injured or disrupted epithelial cell layers, cause further epithelial cell damage, and in many cases, gain access to other tissues or the blood stream. We have previously shown that apical addition of *P. aeruginosa* to MDCK cells mimicked several features of acute pneumonia caused by *P. aeruginosa* in animal models. We have developed a simple, reproducible, rapid and inexpensive genetic screen for *P. aeruginosa*-induced epithelial cell cytotoxicity in MDCK cell culture. This screen was used to isolate isogenic trasposon-tagged non-cytotoxic mutants of a cytotoxic and lung virulent strain of *P. aeruginosa* (PA103). Based on phenotypic and genotypic analysis, the mutants that we have characterized so far can be divided into at least four classes: (i) pili defective, (ii) adherence defective (iii) type III secretion defective and (iv) others. These results suggest that localized cytotoxicity is likely to require several steps and several components, including pili and proteins exported by type III secretion and a novel putative cytotoxin.

These isogenic mutants were used to investigate the relationship between cytotoxicity and invasion of epithelial cells. Although internalization of wild-type cytotoxic strain PA103 was not detectable by standard aminoglycoside exclusion assays, six of the noncytotoxic mutants were invasive. 5/6 of these mutants were defective in secretion of several proteins by type III secretion. Another mutant was specifically defective in the secretion of a 40 kDa and 32 kDa protein. N-terminal sequencing of these two proteins showed no homologs in the DNA and protein data bases. This finding demonstrates that the *Pseudomonas* type III secretion system is crucial in the modulation of both cytotoxicity and invasion, and the lack of secretion of specific target proteins correlates with bacterial invasion of epithelial cells. This finding is of particular interest because other investigators have suggested that there is an inverse relationship between cytotoxicity and invasion; our results demonstrate that a single strain can be (i) non-cytotoxic and non-invasive, (ii) non-cytotoxic and invasive or (iii) cytotoxic. Two models are consistent with our genetic data: either invasion precedes cytotoxicity or invasion and cytotoxicity are mutually exclusive. We are currently utilizing pharmacological and genetic approaches to distinguish beten these two models.

As invasion of host cells followed by their shedding has been postulated to be an important host defense mechanism which may be lost, for example, in cystic fibrosis patients, perturbation of this phenotype may present novel targets for anti-*Pseudomonas* therapies. Moreover, the invasion potential of this bacteria could be subverted for drug delivery.

# FLUORESCENCE MICROSCOPIC VISUALIZATION OF THE MICROTUBULE-DEPENDENT ENTRY OF *CAMPYLOBACTER JEJUNI* 81-176 INTO INT407 CELLS

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*Campylobacter jejuni* is a major cause of bacterial diarrhea worldwide. The pathophysiology of this disease is poorly understood, but current evidence suggests that the ability of *C. jejuni* to invade enterocytes is an important step in pathogensis. Utilizing biochemical inhibitors of eukayotic structures and processes, we have previously reported that *C. jejuni* 81-176 enters cultured human intestinal INT407 cells via a unique process that requires polymerized microtubules, but is unaffected by depolymerization of microfilaments. In contrast, *Shigella, Salmonella*, and *Yersinia* all trigger strict microfilament-dependent entry pathways. The current studies were conducted to obtain direct evidence of the involvement of microtubules in *C. jejuni* internalization into INT407 host cells.

INT407 cells were infected with *C. jejuni* 81-176 at an MOI of 200 for 30 min., 1 hr and 2 hrs. Infected monolayers were reacted with mouse monoclonal anti-α-tubulin antibody and rabbit anti-*Campylobacter* antibody. After washing, the monolayer was then incubated with mouse monoclonal antibodies differentially labeled with Texas Red-X or Oregon Green 514. The stained monolayers were viewed with a Zeiss MC100 fluorescence microscope and a BioRad MRC600 confocal laser scanning microscope.

Both phase contrast and 2D-fluorescence microscopic analyses of INT407 cells infected with *C. jejuni* for 1hr, showed that many bacteria were cell-associated and many cell-bound bacteria appeared to be oriented in parallel with microtubules. Confocal microscopic analyses of infected cell preparations showed again that many cell-bound bacteria were aligned in parallel with microtubules. Further, consistent with co-localization of two components, the green-fluorescent labeled *C. jejuni* appeared as a yellow fusion color when tightly associated with the red-fluorescent labeled microtubules. Using anti-actin antibodies, cell-bound *C.jejuni* were not typically aligned in parallel with F-actin and did not exhibit a fusion color under differential fluorescence labeling of bacteria and microfilaments.Direct fluorescent microscopic analyses of monolayers pretreated with colchicine or cytochalasin D prior to *C. jejuni* addition showed the expected depolymerization of microtubules or F-actin, respectively, but only colchicine blocked *C. jejuni* entry. *Salmonella typhi* was employed here as a F-actin-dependent, microtubule-independent control. Thus, these studies provide additional evidence of the requirement for microtubules in this *C. jejuni* entry mechanism and show the co-localization of *C. jejuni* 81-176 with microtubules during the invasion process.

**THE ROLE OF S FIMBRIAE IN THE PATHOGENESIS OF E.COLI MENINGITIS.** Sheng H. Huang, Ying Wang, Qi Fu, Carol Wass, Linette Linsangan and Kwang S. Kim. Division of Infectious Diseases, Childrens Hospital Los Angeles, University of Southern California School of Medicine, Los Angeles, CA 90027.

*E.coli* is the most common gram-negative organism that causes meningitis during the neonatal period. Our studies have shown that efficient penetration of K1 *E.coli* across the blood-brain barrier is a complex involved in multiple steps of bacteria-host interactions, e.g., S fimbriae for binding to brain endothelium, and several invasion determinants (*ibe7* and *ibe10*) for invasion of brain endothelial cells. Since bacterial meningitis usually develops as a result of hematogenous spread, the important question is how circulating bacteria bind to brain microvascular endothelial cells (BMEC). We have previously shown that S fimbriae are responsible for binding to BMEC and identified the two domains for binding to BMEC, SfaS adhesin for binding to BMEC glycoprotein and SfaA protein for binding to sulfatide. There are two gene clusters encoding S fimbriae, *sfaI*, from uropathogenic *E.coli*, and *sfaII*, from K1 *E.coli* causing meningitis. Partial sequence of *sfaII* locus (*sfa*A,G,S,H) was determined by Hacker et al from a CSF isolate IHE3034. In order to dissect the role of S fimbriae in the pathogenesis of *E.coli* meningitis, we cloned a 16.5 Kb DNA fragment containing the entire *sfaII* from another CSF isolate, RS218 and nucleotide sequencing of *sfaII* gene cluster including *sfa*C,B,D,E,F was accomplished by primer walking. Using a positive selection suicide vector and the cloned DNA, a *sfaA* deletion mutant was constructed. The mutant was found to be unable to invade the blood-brain barrier in vitro. Our preliminary data suggest that S fimbriae play an important role in the pathogenesis of *E.coli* meningitis.

PHYSICAL LIMITS ON *SALMONELLA TYPHI* ENTRY INTO INT407 INTESTINAL EPITHELIAL CELLS.

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*Salmonella typhi*, the cause of human typhoid fever, triggers a microfilament-dependent entry into cultured cells. Our kinetic studies of the invasion process have revealed a strict physical limitation on *S. typhi* Ty2W entry into INT407 human epithelial cells at MOIs≥40. Fluorescence microscopy of acridine orange/crystal violet-stained, time course-infected monolayers showed that all host cells were susceptible to *S. typhi* entry and that internalized bacteria were typically located in 2-4 clusters or foci of infection per host cell. Scanning EM revealed that at early times postinfection, the host cell apical surface contained 2-4 membrane focal aggregates, each containing 1 or more bacteria bound to shortened microvilli. Both scanning EM and TEM analyses showed that at 10-30 min postinfection, the INT407 cell undergoes surface-detectable, cytoskeletal rearrangements. At later times (45-60 min) the eukaryotic cell becomes almost totally denuded of microvilli, and major membrane protrusions/ruffles appear to engulf, via macropinocytosis, bacteria bound in surface focal aggregates. Together, these data provide direct evidence that *S. typhi* are internalized at a limited number (~2-4) of entry sites/receptors on the apical surface of infected INT407 cells, after which further entry is limited. This conclusion was supported by competitive inhibition assays in which a $Km^R$Ty2W strain was inhibited 2-3 logs in entry efficiency by monolayer pretreatment for 30 min with Ty2W. Although the host cell membrane shows major structural alterations by scanning EM at 30-45 min postinfection, preinfection with Ty2W did not reduce the microfilament-dependent, *Yersinia* invasin-mediated entry pathway. In contrast, *S. typhimurium* was efficiently and competitively inhibited by Ty2W, indicating that these 2 serovars recognize the same major INT407 cell receptors. These physical analyses show that *S. typhi* enters INT407 cells by a mechanism that appears very similar to that reported for other *Salmonella spp.* In further studies, preincubation of INT407 monolayers with mouse monoclonal antibodies to the β1 integrin or certain members of this superfamily, reduced invasion ability of *E. coli* HB101(pRI203) harboring the cloned invasin gene by 80%. However, *S. typhi* entry was not reduced even at higher antibody concentrations. These data suggest that the *S. typhi* receptor(s) are different from the "invasin" integrin receptors and are present in limited numbers on INT407 cells.

# THE MANNOSE-SENSITIVE HEMAGGLUTININ (MSHA)-PILUS IS THE RECEPTOR FOR FILAMENTOUS VIBRIOPHAGE 493

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Waldor and Mekalanos recently demonstrated that the *Vibrio cholerae* toxin-coregulated pilus (TCP), a type IV pilus, is the receptor for the filamentous CTX$\phi$ which carries the CTX virulence cassette. We have isolated a filamentous phage from *V. cholerae* O139 strain AJ27-493 ("493") which is inhibitory to all *V. cholerae* O1 El Tor (but not classical) biotype strains isolated before the advent of O139 in India and Bangladesh in 1992. El Tor strains isolated in 1995 after O139 lost its prominence are refractory to inhibition by 493. That TCP was not the receptor for 493 was demonstrated by the sensitivity of strain KHT52 C6706 str $\Delta$*tcpA*10 a derivative of MSHA$^+$, 493 sensitive wild type strain C6706. Evidence that the 493 receptor was, instead, the MSHA type IV pilus was derived from the following studies. Spontaneous MSHA$^-$ mutants (17$^-$ and 26-3$^-$) derived from sensitive MSHA$^+$ parents were not inhibited by 493. Tn-5 insertional mutants of 493-sensitive MSHA$^+$ $\Delta$*ctxAB* from strain JBK70 were insensitive to 493. Strain KHT46, a $\Delta$*mshA1* derivative of C6706, was resistant to 493; sensitivity was restored by *prtc•mshA*1 which expressed MSHA. And, finally, an older MSHA$^+$ spontaneous mutant of O1 classical biotype strain 20-A-11 (aka NIH41 which expressed MSHA variably) and its parent, were both sensitive to 493. Recent isolates of El Tor which are MSHA$^+$ are resistant to inhibition to 493. The reason for this is presently obscure: the MSHA could have a refractory point mutation; the strains could be infected with a phage which confers immunity; or other factors may apply.

We conclude: 1) that MSHA is the receptor for 493; 2) we believe that 493 played a role in the territoriality of O139; 3) we have shown that 493 is competent to transmit genetic information horizontally; and 4) we postulate that 493 could have (or represents a prototype for a filamentous vibriophage which) played a role in the transformation of El Tor to O139.

# IDENTIFICATION OF *ESCHERICHIA COLI* GENES EXPRESSED DURING SEPTICEMIA.

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The essential requirement of a pathogen is to evade the host's immune system and adapt to environmental and nutritional changes. Pathogenic bacteria are thought to express specific sets of genes in the host for successful infection. In this study we sought to identify genes of a septicemic *E. coli* that are specifically derepressed in a murine model of septicemia. To identify genes induced during septicemia we used a chloramphenicol acetyl transferase (*cat*) gene as reporter. A genomic library was prepared in the plasmid vector pKK232-8 that carries a promoterless *cat* gene adjacent to a cloning site. The genomic library was electroporated into wild type *E. coli* strain. A group of chloramphenicol treated mice were then challenged with the genomic library to select for clones expressing chloramphenicol acetyl transferase in infected mouse. Bacterial clones were recovered from mouse liver. *In vitro* chloramphenicol sensitive clones were identified by replica plating and used for a second round of *in vivo* selection in chloramphenicol treated mouse. 700 clones were recovered from mouse livers that exhibit an *in vitro* chloramphenicol sensitive phenotype. Seventeen of the identified clones were sequenced, five clones contain uncharacterized ORFs of *E coli*. Other clones contained the following genes: *clpB, prlC, aroA, bglC, dcuA, narB* and a gene of transcription repair coupling factor (TRCF).

To confirm that the reporter gene was expressed *in vivo* by these clones during infection, *cat*-specific mRNA was detected by RT-PCR from RNA extracted from infected mouse liver. In addition, *in vivo* induced clones and the wild type *E. coli* strain were used to challenge two groups of chloramphenicol treated mice. Infection was followed over a period of 72 hours. After 72 hours the wild type strain was completely eliminated from the livers of chloramphenicol treated mice, whereas $6X10^6$ cells were recovered from mouse liver challenged with *in vivo* induced clones. Further studies are being carried out to characterize and evaluate the importance of the *in vivo* induced clones for pathogenesis.

**EVIDENCE FOR AN ASSOCIATION BETWEEN PORE FORMATION BY *LEGIONELLA PNEUMOPHILA* AND INTRACELLULAR REPLICATION**

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*Legionella pneumophila*, the cause of Legionnaires' pneumonia, replicates within phagocytic vacuoles. After internalization by macrophages, it occupies a replicative phagosome that bypasses the endocytic pathway and is bound by endoplasmic reticulum. An association between cytotoxicity and intracellular growth is demonstrated. Cytotoxicity for primary macrophages and red blood cells was dependent on *dotA* as well as a variety of other loci known to be required for intracellular growth and targeting to the replicative phagosome. Cytotoxicity occurred rapidly, was evident at low multiplicities of infection, and required close association of the bacteria with the eukaryotic cell. The rapid osmotic lysis and differential cytoprotective ability of polyethylene glycols with a molecular weight greater than or equal to 3350 was consistent with the insertion of a pore less than 3 nm in diameter into the plasma membrane.

Therefore, the apparent ability of *L. pneumophila* to insert a pore into the eukaryotic membrane correlated with the ability to properly target and replicate within a macrophage. The putative pore may serve to initiate proper targeting or perhaps allow passage of another bacterial product into the mammalian cell.

## GENETIC ANALYSIS OF TCPA PILIN STRUCTURAL AND FUNCTIONAL DOMAINS

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The toxin coregulated pilus (TCP) is expressed by *Vibrio cholerae* O1 and O139 as part of the ToxR virulence regulon and is required for bacterial colonization of the human intestine. The TCP structure is assembled as a polymer of repeating subunits of TcpA pilin. Several immunological and structural studies have suggested that the C-terminal disulfide loop region of TcpA is likely to be surface exposed along the pilus fiber. In the present study, this region was further characterized by converting each codon specifying a charged amino acid residue to an alanine codon. After exchanging each mutation for the wild-type gene on the O395 chromosome, various properties of TCP were examined. These included pilus morphology, bacterial autoagglutination, and colonization in the infant mouse cholera model. The mutations revealed that several residues, including the two Cys residues that delineate the disulfide loop, are critical for pilin stability. The phenotypes of mutants that produced stable pilin revealed that the disulfide loop region can be divided into two domains. The N-terminal portion of the region appears to be critical for bacterial autoagglutination and colonization since the majority of mutations causing alterations to this region strongly affected these properties. In contrast, most mutations resulting in alterations toward the C-terminal portion of the disulfide loop region had little effect on these properties. The region most affected by the mutations correlates to a potential protective epitope based on the passive immunity conferred by antibodies raised against a synthetic peptide corresponding to the region. The mutations also demonstrated a high correlation between bacterial autoagglutination and colonization, suggesting that the ability of TCP to promote interaction between bacterial cells may be relevant to its colonization function. One mutation was identified that dramatically altered TCP morphology from a straight fiber to a curly structure. This mutant no longer autoagglutinated and was greatly impaired for colonization. A spontaneous revertant that regained TCP associated function and morphology was determined to be an intragenic suppressor mutation located in a codon corresponding to a residue within the N-terminal hydrophobic helical domain of the pilin. The location of this suppressor suggests interaction between the N-terminal hydrophobic helical domains of the pilin subunits within the pilus fiber.

**PERIPLASMIC SUPEROXIDE DISMUTASE IN *SALMONELLA*: A NOVEL, HORIZONTALLY-ACQUIRED, CONTRIBUTOR TO VIRULENCE.**

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Copper- and zinc-cofactored superoxide dismutase [Cu,Zn-SOD] has been found in the periplasm of an ever-widening range of bacteria but its biological function is not known. Here we report the cloning and characterization of *sodC*, encoding [Cu,Zn]-SOD, from *Salmonella typhimurium*. The predicted protein sequence demonstrates unexpectedly that the mature peptide shows only 58% identity to *Escherichia coli* SodC, in contrast to the usual approx. 95% identity of authentic *S. typhimurium/E. coli* orthologs. Characterization of the *sodC* locus in *S. typhimurium* has established that the gene lies at a different map position from that predicted based on the *E. coli sodC* locus, on a monocistronic operon flanked by genes encoding proteins similar to minor tail protein L and the Ail-like protein Lom of bacteriophage lambda. This suggests that *sodC* may have been acquired by Salmonella through phage-mediated transfer from an unidentified donor species.

To define the role of [Cu,Zn]-SOD in Salmonella biology and pathogenesis, we have created knock-out mutations at the *sodC* locus in several serovars ( *S. typhimurium*, *S. choleraesuis* and *S. dublin*). Compared to wildtype, *sodC* mutants showed reduced lethality in a mouse model of oral infection and persisted in significantly lower numbers in livers and spleens following intraperitoneal infection, suggesting that [Cu,Zn]-SOD plays a role in protection of *Salmonella* against host defenses. To clarify the molecular basis for these observations, we have assessed resistance of wildtype and *sodC* mutant organisms to the bactericidal effect in vitro of superoxide generated in the extracellular environment by enzymatic oxidation of xanthine. Mutant organisms were significantly more vulnerable, suggesting that periplasmic SOD may contribute to bacterial virulence by enhancing resistance to host phagocyte-derived toxic oxygen species. However at the cellular level we were unable to distinguish a difference between wildtype and mutant organisms in survival *ex vivo* within J774 mouse macrophages. Set against the clear difference in liver/spleen infection, this result may reflect the suggested hierarchical nature of the capacity of different macrophage lines to kill *Salmonella*, highly efficient J774 cells overwhelming the proposed protective effect of periplasmic SOD.

In models of epithelial infection, SodC did not appear to play a critical role. In a ligated ileal loop model of enteric infection, *sodC* mutants were unimpaired in their ability to invade the mucosa or to induce inflammatory/secretory responses.

We conclude that [Cu,Zn]-SOD plays a role in the pathogenesis of systemic *Salmonella* infections subsequent to epithelial invasion, conferring a degree of resistance to host defenses mediated by toxic oxygen species.

# INVASIVE ADENYLATE CYCLASE TOXIN OF *BORDETELLA PERTUSSIS* : A VEHICLE TO DELIVER FOREIGN EPITOPES INTO ANTIGEN-PRESENTING CELLS

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*B. pertussis*, the causative agent of whooping cough, produces a virulence-associated, calmodulin-dependent adenylate cyclase toxin (CyaA). It is a 1706-residue-long protein, able to invade a large number of eukaryotic cells and to deliver its N-terminal catalytic domain (400 residues) directly into the cytosol through the cytoplasmic membrane. We have previously identified within the primary structure of CyaA several permissive sites which tolerate insertion of peptides without altering the catalytic and invasive activities of the toxin. Choosing one of the permissive sites within the catalytic domain of CyaA, we constructed a number of recombinant toxins and showed that they can accomodate insertions up to 150 amino acid residues without interfering with the different functional activities.

We constructed recombinant CyaAs carrying $CD8^+$ T cell epitopes either from the nucleoprotein of the lymphocytic choriomeningitis virus (LCMV) or from the V3 region of HIV- gp120. We showed that mice immunized with these toxins developed epitope-specific cytotoxic responses, mediated by $CD8^+$ cytotoxic T lymphocytes (CTL) restricted by the major histocompatibility complex (MHC) class I molecules, thus showing that the epitopes were produced in the cytosolic compartment of the presenting cells. Moreover, mice immunized with a recombinant CyaA toxin carrying the LCMV epitope were fully protected against intracerebral challenge with live LCMV, which killed control mice within seven days. Protection was associated with total virus clearence, was abrogated after depletion of $CD8^+$ T cells and was independent of the adenylate cyclase activity of the toxin. Indeed, a genetically inactivated invasive CyaA carrying the LCMV epitope, protected mice against challenge.

This study represents the first demonstration that a genetically detoxified bacterial toxin, carrying a viral CTL epitope, can stimulate efficient protective immunity. It also shows that the *B. pertussis* adenylate cyclase toxin is a versatile and flexible protein, able to accomodate large peptide insertions that could be delivered to various eukaryotic cells. By inserting peptide epitopes at single or multiple sites one might envisage the use of these recombinant proteins as vaccine components or as targeted immunotoxins.

TCPJ-MEDIATED PILIN PROCESSING AND EXPORT: MUTATION AND SUPPRESSOR ANALYSES.

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The toxin coregulated pilus (TCP) of *Vibrio cholerae* is a type 4 pilus that is essential for the disease pathogenesis of *Vibrio cholerae*. Processing of the precursor TcpA pilin subunit by the prepilin peptidase, TcpJ, is a required step in its secretion and assembly into the pilus fiber. Type 4 pilins share extensive homology in the region surrounding the peptidase processing site. In order to further examine the processing constraints within this region, random mutagenesis was performed on the corresponding portion of *tcpA*. Defective processing or altered pilin migration resulted from changes at positions -3, -1, and +5 with respect to the processing site. A comprehensive analysis of these positions was performed using amber mutations engineered into the corresponding codons in conjunction with a tRNA suppressor collection. All the replacements of the invariant Gly residue at position -1 that were tested resulted in a processing defect, whereas no change was found at the invariant Glu at +5 that completely prevented processing. Alterations at this Glu position often resulted in aberrant migration of the mature pilin. The -3 Gln position was found to be able to retain processing if Glu or Phe were substituted, but nine other changes at this position were unable to support complete processing. A TcpA-LamB hybrid protein was constructed by replacing the signal peptide of LamB with the TcpA signal peptide. Introduction of non-processing *tcpA* mutations into this construction were found to cause a severe growth defect similar to that described for LamB processing mutations (Carlson and Silhavy, J. Bact. 175: 3327). Interestingly, some combinations of *tcpA* mutations defective for processing were identified using the TcpA-LamB fusion that restored normal growth and processing. This system is currently being used to attempt to identify residues of TcpJ that interact with prepilin to restore processing of defective TcpA-LamB hybrid proteins.

# A COMPARISON OF CTB AND CTA2 SUBUNITS AS RECOMBINANT FUSION PROTEINS FOR CARRIAGE OF CHLAMYDIAL T-CELL AND B-CELL EPITOPES

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For the purpose of producing a mucosal immunogen able to induce a protective immune response against sexually transmitted Chlamydial infection a gene fusion was constructed in which a 55 amino acid peptide carrying both conserved T-cell and B-cell epitopes from the major outer-membrane protein (MOMP) of *Chlamydia trachomatis* was linked to the amino terminus of the cholera toxin B subunit (CTB).
The resulting gene product was expressed in *E.coli* where the presence of the added peptide was confirmed by GM1-ELISA and western blot using a monoclonal antibody (mAb) against the chlamydial B-cell epitope. A gene fusion of the same peptide was made to the amino terminus of the A2 moiety (CTA2) of the cholera toxin A subunit (CTA). This fusion was co-expressed in *E.coli* with native cholera toxin B subunit (CTB). Assembly of the CTA2 gene fusion with CTB was confirmed by GM1-ELISA.
Initial results indicated that whereas it was possible to fuse large peptides to CTA2, the yields of the chlamydial CTA2-fusion proteins were considerably lower than those obtained with the corresponding CTB fusion. Upon further analysis we concluded that fusion of peptides to CTA2 lead to inefficient assembly of the products, since a large proportion of pentameric CTB (>90%) did not associate with CTA2 fusion peptides.
More efficient assembly into holotoxin could be obtained ,however, if the expression levels of the CTA2 fusion were increased relative to CTB by alteration of the ribosome binding site and by the use of other signal peptide sequences. Furthermore, increasing the size of the fusion peptide by adding several copies of the same B cell epitope to the CTA2 subunit did not improve its ability to assemble with CTB. Comparison of the immunogenicity of the hybrid proteins in animals is in progress.

## SEROTYPE-SPECIFIC GENE CASSETTES IN THE FOOD-BORNE PATHOGEN, LISTERIA MONOCYTOGENES

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Listeria monocytogenes serotype 4 has been implicated in ca. 40% of sporadic cases of listeriosis and in most epidemics of the disease. We have identified two serotype-specific gene cassettes in the genome of serotype 4 strains. The cassettes appear to occupy separate loci, and are found in no other serotypes of Listeria monocytogenes. Interestingly, however, sequences homologous to both cassettes are found in certain strains of the non-pathogenic Listeria, L. innocua.

Insertional inactivation of one of these cassettes rendered the bacteria non-invasive and phage-resistant. The cassette was able to complement the mutant phenotypes in trans. Each cassette is flanked by sequences which are conserved among other serotypes. Southern blots suggest that the sequences may have been transferred to this serotype of Listeria from a another source, likely to be outside the genus, perhaps through phage-mediated transfer(s).

# STRATEGIES USED BY *AEROMONAS HYDROPHILA* TO INVADE CARP EPITHELIAL CELLS.

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*Aeromonas hydrophila* is the causal agent of motile aeromonad septicemia, a major fish disease affecting warm-water aquaculture throughout the world. The *in vitro* interactions of *A. hydrophila* with epithelioma papillosum cells of carp (EPC) were studied. All the virulent strains invaded and multiplied inside EPC cells. Morphological changes were observed in infected fish cells and could be classified into 3 different stages (Stage I, II & III). Presence of metabolically active bacteria was required for invasion and the extracellular products released from the bacteria played a minor role in the morphological changes of EPC cells we observed. On the other hand, avirulent strains did not invade, multiply or induce cytopathic effects in the EPC monolayers.

Rearrangement of microfilaments (F-actin) was observed as local polymerization in Stage I and massive reorganization in Stage III of infection. Pretreatment of fish cells with the microfilament inhibitors such as cytochaslasin D induced similar effect. There were little if any rearrangements of intermediate and microtubule filaments during bacterial entry (Stage I and II). The invasion of *A. hydrophila* into the EPC cells was further studied by screening signal transduction inhibitors. Genistein, a tyrosine kinase inhibitor, postponed invasion of *A. hydrophila* into host cells while staurosporine, a protein kinase C inhibitor, and sodium orthovanadate, a protein tyrosine phosphatase inhibitor, accelerated the invasion. These results suggest that rearrangement of microfilaments and activation of signaling pathways may mediate *A. hydrophila* entry into EPC cells.

THE ROLE OF MICROBIAL ENDOCRINOLOGY IN THE PATHOGENESIS OF INFECTIOUS DISEASE

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A new theory has been proposed which involves the interaction of host neuroendocrine factors with pathogenic bacteria. Upon entrance into a host, pathogenic microorganisms encounter within the gastrointestinal system a myriad of neuroendocrine hormones, one of the principal ones being the catecholamine norepinephrine (NE). The possibility that exposure to NE may serve as a host environmental signal governing growth of pathogenic bacteria and production of virulence factors was investigated.

In vitro and in vivo methodology was employed to demonstrate neuroendocrine-bacterial interactions. In vitro culture consisted of low initial inocula of ETEC or EHEC strains into a serum-based medium supplemented with various catecholamines to more closely approximate in vivo conditions. Virulence factor production was assayed by ELISA. Medium generated from the growth of NE grown bacteria (conditioned medium) was also examined for the presence of an autoinducer of growth in an assay system which quantitated the growth of fresh bacteria in non-NE containing medium to which serially diluted conditioned medium was added. In vivo neuroendocrine-bacterial interactions were examined using a neurotoxin-induced model of trauma in which the administration of 6-hydroxydopamine (6-OHDA) resulted in a reversible destruction of adrenergic neurons in the gastrointestinal tract and release of NE.

The in vitro growth of ETEC and EHEC in NE supplemented medium was increased several logs as compared to controls. Production of Shiga-like toxins in E. coli O157:H7 was increased on a protein equivalent basis 160-fold in NE cultures as compared to controls. Elaboration of the K99 pilus adhesin in the ETEC B44 strain was increased 1600-fold in NE cultures. The ability of NE to induce growth and production of virulence factors was shown to be non-nutritional, possibly receptor-mediated, in nature. Further, in NE cultures the production of an autoinducer of growth capable of stimulating the growth of fresh non-NE exposed bacteria was demonstrated. In neurotoxin-treated mice a 5 log increase in the cecal level of indigenous E. coli was observed 24 following 6-OHDA administration. The specificity of the NE-induced E. coli growth was demonstrated with the use of the catecholamine uptake blocker desipramine. Time-dependent regeneration of adrenergic neurons resulted in a restoration of normal cecal levels of E. coli.

The results of both in vitro and in vivo studies have shown that the growth of pathogenic and indigenous bacteria may be greatly increased by direct interaction with the host's neuroendocrine hormones, particularly the catecholamines. Additionally, the production of virulence-related factors were also increased due to exposure to NE. One possible mechanism governing early log phase bacterial growth involved the production of a bacterial autoinducer of growth in NE grown ETEC and EHEC that was not observed in standard "rich" microbiological media such as tryptic soy broth.

It is therefore suggested that the host's neuroendocrine hormones, particularly as encountered within the highly innervated gastrointestinal tract, may serve as a type of environmental cue by which microorganisms may sense their surroundings and thereby initiate pathogenic processes.

# SHIGELLA TREATED WITH POLYMORPHONUCLEAR LEUKOCYTE EKSTRACTS IS ALTERED IN ITS CAPACITY TO INDUCE APOPTOSIS IN J774 MACROPHAGES.

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Shigellae are invasive bacteria that cause dysentery, predominately in the developing world, where it is often fatal for young children. A crucial property in the pathogenesis of shigellae is the microorganism's capacity to invade the cytoplasm of eukaryotic cells and elicit an immune response. We are studying interactions between Shigella and polymorphonuclear leukocyte (PMN) and find that rabbit PMN efficiently kill invasive wild-type strain of Shigella flexneri (M90T) and a plasmid-cured noninvasive derivative (BS176). Bacterial killing by PMN extracts is dose dependent, is partially inhibited by antibodies to the bactericidal/permeability-increasing protein (BPI) and can be reversed by BSA. Whereas wild - type Shigella escapes from the phagosome to the cytoplasm in epithelial cells and macrophages and induces apoptosis in the later cells, these bacteria become trapped in the phagosomes of PMN as visualized with electron microscopy and do not cause specific apoptosis in these cells. Interestingly, wild type Shigella treated with PMN extract still induces apoptosis in macrophages but the entry into programmed cell death of infected macrophages is delayed as compared to macrophages infected with the untreated Shigella. The ability of PMN to trap and kill invasive shigellae within the phagolysosome suggests that these cells are likely to play an important role in the resolution of dysentery caused by this pathogen.

# CHARACTERIZATION OF THE MANNOSE-SENSITIVE HEMAGGLUTININ OF *VIBRIO CHOLERAE*

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The mannose-sensitive hemagglutinin (MSHA) of *Vibrio cholerae* is a member of the family of Type IV pili. Type IV pili are found on a variety of Gram-negative bacteria and have multiple roles as host cell attachment factors, bacteriophage receptors and as mediators of DNA uptake. MSHA is expressed specifically on the surface of the El Tor biotype of *V. cholerae*. Although the MSHA pilus is not required for colonization of the intestinal epithelium, recent studies indicate that MSHA is the receptor for the filamentous vibriophage, 493 (Jouravleva *et al.*, this meeting). The gene locus required for the assembly and secretion of the pilus has been localized to a ~20 kb region of the *V. cholerae* chromosome. Sixteen genes have been identified within this region that are required for the MSHA phenotype. Several of the predicted gene products show homology to bacterial proteins involved in extracellular secretion. A cluster of genes which encode distinctive Type IV structural pilin subunits reside just downstream of the secretory homologs. Promoter analyses suggest that two separate promoters are required to drive the expression of the structural and secretory loci. Examination of specific cDNAs from these two loci indicate the position of the promoters relative to the start of transcription. The genes flanking this pilus biogenesis locus encode proteins which show homology to YhdA and MreB of *E. coli*, the latter of which is involved in rod shape formation. In *E. coli*, the *yhdA* and *mreB* genes are adjacent to each other in the chromosome. The finding that the MSHA locus lies between these two *E. coli* gene homologs and that it is flanked by a 7 base pair direct repeat suggests that the MSHA pilus gene cluster may have been acquired by *V. cholerae* as a transposon. Since no transposase appears to be associated with the locus, future studies will address the nature of this unique genetic element.

REGULATORY FEATURES OF BUNDLE-FORMING PILUS EXPRESSION IN ENTEROPATHOGENIC *E. COLI* (EPEC).
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In EPEC, the *bfpA* gene codes for the structural subunit of the bundle-forming pilus (BFP), which is necessary for the localized adherence phenotype and is located on the EAF (EPEC-adherence factor) plasmid. *bfpA* is regulated by the growth phase, temperature and ammonium concentration, and its expression requires the product of the *bfpT* gene (also called *perA*), a member of the AraC family of transcriptional activators.

*bfpT* expression is autoregulated, as a *bfpT* reporter gene fusion was not expressed in an EAF-minus nor in a non-EPEC strain, unless a functional *bfpT* was present. Furthermore, *bfpT* expression was also regulated by the growth phase, temperature and ammonium concentration, suggesting that *bfpA* regulation is, at least in part, dependent on the cellular levels of BfpT, in response to environmental cues.

Moreover, site-directed and PCR-random mutagenesis, as well as deletion analysis of the *bfpA* upstream regulatory region, demonstrated that the sequence between -84 to -40, showing direct- and inverted-repeats, contains the cis-acting elements that mediate BfpT-dependent expression and regulation of *bfpA*. They also supported the assignment of the -10 and -35 promoter regions. Interestingly, this region shares a 70% identity with a 47 bp-long AT-rich tract, located upstream of the *bfpT* gene, which is essential for *bfpT* autoregulation.

In summary, these results are the basis towards identifying cis-acting elements involved in the BfpT-dependent regulation of other virulence factors in EPEC.

*These authors contributed equally to this work

# ADAPTIVE MUTATIONS WITHIN *SALMONELLA TYPHIMURIUM* INVOLVING A RESOLVASE-LIKE GENE AND THE *SPV* OPERON.

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*Salmonella typhimurium* is a Gram-negative enteroinvasive organism which invades and proliferates in the small intestine and Peyer's patches, causing gastroenteritis. The nontyphoid Salmonella serovars *S. typhimurium, S. choleraesuis, S. dublin, S. enteritidis, S. gallinarum,* and *S. pullorum* harbour large plasmids required for the production of lethal systemic infection in experimental animals. These virulence plasmids vary considerably in size (50-100 kb) and in overall nucleotide sequence, however the core virulence genes designated *spv* (for Salmonella plasmid virulence) are highly conserved among all serovars.

The *spv* coding region consisting of the regulatory gene *spvR* and the structural genes *spvABCD* spans approximately 6 kb. It has been shown that *spvABCD* form a single operon, transcribed from promoters upstream from *spvA*. The *spvR* gene is located directly upstream of the *spvABCD* operon and is transcribed as a distinct message in the same orientation as *spvABCD*. The SpvR protein is required for transcription of *spvABCD*.

A strain of *Salmonella typhimurium*, CJD671, contains a MudJ fusion to the structural gene, *spvB,* of the *spv* operon. When colonies of CJD671 are aged on MacConkey-Lactose indicator medium, mutants arise that express the *spv* operon at a higher level. Several of these mutants have a deletion of a fragment of DNA directly upstream of the *spvR* gene resulting in the close association and transcriptional read-through from a resolvase-like gene into the *spv* operon. The inability of these mutations to be induced under non-selective stress suggests that they may be adaptive mutations. The occurrance of these deletions *in vivo* is currently being investigated.

CONTROL OF ACETYLATION OF GROUP E1 SALMONELLA ENDOTOXIN BY BACTERIOPHAGES, by Michael McConnell, Brent Butts, Kristin Abell Johnson, Brent Whitehead, Jeanette Eastis, David Mills, Suzanne Norton, Francesca Conte, David Brock and Vitruc Tran, Departments of Biology and Chemistry, Point Loma Nazarene College, San Diego, CA 92106

Infection of Group E1 Salmonellae bacteria by bacteriophages c341 and Epsilon 15 causes conversion of the O-polysaccharide portion of the endotoxin (lipopolysaccharide) of the host cell. Phage c341's effect is quite subtle; namely, loss of O-acetyl groups from galactose sugars in the repeat unit of the O-polysaccharide. Phage Epsilon 15 exerts a more dramatic effect, causing the repeat units of the O-polysaccharide to change from being alpha-glycosidically-linked and O-acetylated to being beta-linked and non-acetylated. Evidence from the 1960s suggested that Epsilon 15 causes loss of acetyl groups by producing a transacetylase repressor (TR) protein that blocks expression of the host cell gene coding for the O-polysaccharide transacetylase enzyme.

We have amplified and cloned a 0.5 kbpr region of the Epsilon 15 genome that expresses the TR conversion function of Epsilon 15. Hybrid plasmids carrying the 0.5 kbpr insert block acetylation of O-polysaccharide in vivo and yield a 6300 dalton polypeptide in an in vitro transcription/ translation system. The PCR primers used for amplifying the 0.5 kbpr Epsilon 15 DNA product also yield a DNA product of identical size and function when phage c341 DNA is used as the "seed" DNA in the PCR reaction. The DNA sequence of the cloned 0.5 kbpr Epsilon 15 DNA fragment contains several different open reading frames (OFRs) that could code for polypeptides with molecular weights of about 6300 daltons. We have narrowed the number of possible repressor gene ORFs to one through the use of additional PCR primer combinations that permit amplification and cloning of selected smaller portions of the 0.5 kbpr insert. The inferred transacetylase repressor polypeptide is small (62 amino acids) and slightly basic (pI = 7.98), but displays no obvious resemblance to other known repressor proteins.

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SALMONELLA TYPHIMURIUM REQUIRE APICAL EPITHELIAL ATTACHMENT, BUT NOT INTERNALIZATION, TO INITATE SIGNALS GOVERNING NEUTROPHIL MIGRATION

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Modeling *Salmonella typhimurium* (*St*)-epithelial interactions *in vitro* has led to the realization that epithelial cells are crucial in orchestrating neutrophil (PMN) responses. However, the signal cascade(s) which govern this complex response have not yet been described. Thus, we sought to identify the primary component of *St*-epithelial interactions required for initiating early transepithelial signals to PMN. For this purpose we utilized a polarized model of human intestinal epithelia (T84 cells), peripheral blood PMN, and *St*. We first established whether *St* could induce transepithelial signals to PMN from either apical or basolateral membrane domains. Despite comparable conditions of surface exposure (100 bacteria/epithelial cell, 1 hr, 37oC), only *St* exposed to apical surface membrane domains were able to induce the appropriate signals compulsory for PMN transepithelial migration (8.84±2.00 vs. 0.744±0.420 for *St*-induced PMN transmigration for apical vs. basolateral exposure, respectively [$p<0.01$] ). Such data suggest apical restricted *St* associations may be crucial and determine transepithelial signal cascades to PMN. Further studies indicate that a sustained population of apically associated *St* is essential and sufficient for promotion of transepithelial signals. For example, *St* colonized monolayers were treated with gentamicin (GM) prior to the addition of PMN in order to eliminate surface attached *St*. GM treatment nearly ablated the subsequent PMN transmigration response; which could not be explained by GM treatment alone (15.12±2.20 vs. 2.50±1.10 x $10e^4$ for *St*-induced PMN transmigration in absence vs. presence of GM, respectively [$p < 0.01$] ). Additionally, despite that cytochalasin D treatment successfully prohibited *St* entry into epithelial cells without altering surface attached bacteria, the ability of *St* to induce transepithelial signals to PMN was surprisingly upregulated (10.02±2.1 vs. 16.5±2.67 x $10e^4$ for *St*-induced PMN transmigration in absence vs. presence of cytochalasin, respectively [$p < 0.01$] ). Likewise, *St*-induced basolateral secretion of interleukin-8 (IL-8) was also upregulated (1 ng/ml vs. 5.5 ng/ml IL-8 in the absence and presence of cytochalasin, respectively [$p<0.01$] ), yet PMA-induced IL-8 secretion was not enhanced. In summary, preferential *St*-apical epithelial interactions requiring attachment but not internalization are essential for initiating the signal cascades which govern neutrophil transepithelial migration.

EFFECTS OF *PSEUDOMONAS AERUGINOSA* EXOENZYME S ON EUKARYOTIC CELL FUNCTION. Eileen M. McGuffie, Timothy S. Vincent, Joan C. Olson. Medical University of South Carolina, Charleston, SC 29425.

Exoenzyme S (**ExoS**) is secreted from *Pseudomonas aeruginosa* and like many bacterial toxins has an ADP-ribosyltransferase activity. Although animal studies suggest that ExoS contributes to *P. aeruginosa* virulence, its targets and mode of action in the mammalian host remain unknown. Difficulties in understanding the function of ExoS in vivo have related to the lack of development of a system that allowed the cellular effects of ExoS to be detected and analyzed. These difficulties are now believed to reflect the type III mechanism of secretion of ExoS from *P. aeruginosa*. Based on the premise that contact between the bacterium and target cell is required for the translocation of proteins via type III secretion, our laboratory, in collaboration with Dr. Dara Frank, developed a bacterial/eukaryotic cell co-culture system that allowed assessment of an effect of ExoS production by bacteria on eukaryotic cell function. Using this co-culture system, ExoS producing *P. aeruginosa* strain 388 bacteria were found to cause a significant decrease in cellular proliferation and viability when compared to that of an isogenic strain, 388Δ*exoS*, which lacks the *exoS* structural gene. Six of seven cell types examined in co-culture studies, including a fibroblastic, an epidermoid, a colon carcinoma and three prostate tumor lines showed significant sensitivities to the effects of ExoS. In comparison, normal human epithelium derived from kidney proximal tubules appeared relatively resilient to the effects of ExoS, with this resilience increasing when cells were allowed to grow to confluency and form tight junctions.

To gain further understanding of the cellular mechanism associated with sensitivities to the effects of ExoS, the co-culture system was used to examine the efficiency of ExoS ADP-ribosylation of Ras in different cell types. Ras modification, as assayed by a shift in molecular mass, was detected following exposure to strain 388, but not strain 388Δ*exoS*, and was more efficient in cell lines with increased sensitivity to the effects of ExoS producing bacteria. It can be concluded from these studies that the contact dependent delivery of ExoS resulted in the cellular modification of Ras and that the efficiency of Ras modification directly correlated with effects of ExoS on cell growth.

The Influence Of Growth Temperature And Osmolarity On Lipopolysaccharide And Virulence Of *Aeromonas Hydrophila* From Different Serogroups.

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We previously reported that the growth temperature influences the composition of lipopolysaccharide (LPS), being smooth (presence of O-antigen) at 20°C and rough at 37°C always on low osmolarity, on strains of *Aeromonas hydrophila* from serogroup O:34. These changes in LPS correlates with a change on thevirulence of these strains in a fish or mice animal model. The presence of the O-antigen decrease the LD50 for these strains in both animal models. Recently, we reported that when the osmolarity increases at 37°C, these strains produced an LPS smooth and also became more virulent in the animal models tested. We decided to test in these results were limited to the strains of these serogroup (O:34), or may be extended to other serogroups. By using at least three different strains of each from the different 44 serogroups initially defined for mesophilic *Aeromonas* sp., we found that strains from serogroups O:13, O:33 and O:44 showed a similar situation that the described for serogroup O:34, while no differences were observed on strains from the rest of all the other serogroups. It is clear that strains of serogroups O:13, O:33 and O:44 are affected on their LPS by the same environmental regulation as strains from serogroup O:34 (presence of antigen O when they grow at low temperature independently of osmolarity or at 37°C with high osmolarity) and the presence of these O-antigens increase the virulence of these strains in animal models.

# CELLULAR STUDIES ON TRAFFICKING OF THE *LEGIONELLA PNEUMOPHILA* PHAGOSOME

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*L. pneumophila* is a bacterial respiratory pathogen that can grow within human alveolar macrophages. Phagosomes containing *L. pneumophila* are not routed to a degradative compartment inside macrophages, instead, the *L. pneumophila* vacuole becomes a unique niche that supports bacterial growth called the replicative phagosome. Our lab is interested in the molecular events that regulate *L. pneumophila* phagosome trafficking and thereby lead to replicative phagosome formation. The *L. pneumophila* DotA protein is a virulence determinant required for intracellular growth. Indirect immunofluorescence microscopy has demonstrated that *L. pneumophila dotA* mutants are unable to impede early intracellular fusion events between the phagosome and late endosomes. These data indicate that the inability to grow within macrophages is due to improper targeting of the membrane-bound compartment harboring *dotA* mutant bacteria. Therefore, a DotA-dependent signal must be transduced from the bacteria to the macrophage in order to alter intracellular trafficking of the phagosome. To investigate the nature of this signal, mouse bone marrow-derived macrophages were infected with either wild type *L. pneumophila* or an isogenic *dotA* mutant strain and then fed a suspension of heat killed *Saccharomyces cerevisiae*. Indirect immunofluorescence microscopy was used to determine whether phagosomes containing the yeast cells were subject to differential trafficking in macrophages infected with wild type *L. pneumophila* when compared to macrophages that first ingested *dotA* mutant bacteria. The yeast particles were found in fused lysosomal compartments in macrophages infected with an *L. pneumophila dotA* strain, as well as in macrophages infected with wild type *L. pneumophila*. The behavior of macrophages infected with *L. pneumophila* was observed using time-lapse video microscopy. These studies reveal that formation and maturation of macropinosomes is unaffected in macrophages infected with wild type *L. pneumophila*. These data show that *L. pneumophila* do not have a global effect on intracellular trafficking of endocytic compartments in the host cell. Thus, the DotA-dependent signal that alters trafficking of the *L. pneumophila* phagosome is *cis*-acting suggesting that the signal is not propagated by a toxin that is able to diffuse freely within the host-cell cytoplasm.

## *IN VIVO* INDUCED GENES OF *ACTINOBACILLUS PLEUROPNEUMONIAE.*

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*Actinobacillus pleuropneumoniae* (APP) is a gram negative rod belonging to the Family *Pasteurellaceae* and the causative agent of pleuropneumonia in swine. To aid in the identification of genes involved in the pathogenesis of APP infection, we have designed an *in vivo* expression technology (IVET) system to identify APP gene promoters that are specifically induced *in vivo* during infection. A functional APP IVET system requires a defined biochemically attenuated APP mutant, the wild type biochemical gene(s) from a different source that can complement that mutation, a readily quantifiable reporter gene with no background in APP, and a delivery vector. We have constructed an APP mutant with a chromosomal deletion in the operon required for synthesis of riboflavin that fails to survive *in vivo* in experimentally infected swine. When the *ribBAH* genes from *Bacillus subtilis* were cloned into this Rib- mutant under the control of a functional APP promoter, both the ability to grow without riboflavin and virulence were restored. We have shown that the *luxAB* genes from *Vibrio harveyi* can be used to monitor gene expression in APP and that APP is not natively bioluminescent. Our APP-IVET promoter-trap vector (pTF86) contains, in sequence, the T4 terminator, a unique *Bam*HI site, a promoterless copy of the *B. subtilis ribBAH* genes, and a promoterless copy of the *V. harveyi luxAB* operon, in the *E. coli*-APP shuttle vector pGZRS19. A plasmid library was constructed by cloning partial *Sau*3A fragments of APP chromosomal DNA into the *Bam*HI site in pTF86 and was transformed by electroporation into the APP Rib- mutant. Pigs were infected by endobronchial inoculation with pools of 300-400 transformants and surviving bacteria isolated from bronchoalveolar lavage fluid and lung tissue at 12-24 hours post-infection. Only those transformants containing cloned promoters active *in vivo* should have survived due to expression of the cloned *ribBAH* genes. Isolates recovered from infected pigs were screened for *lux* expression *in vitro*. Isolates that survived *in vivo*, but which minimally express Lux activity *in vitro,* should contain cloned promoters that are specifically induced *in vivo*. We have analyzed 1800 promoter clones (~10% of the number needed for a representative library) and have identified six clones containing promoters that are induced *in vivo*. Two of these clones have been putatively identified by amino acid sequence homology as the *secE-nusG* operon and the *mrp* gene. Construction and analysis of additional promoter clones is in progress. This is the first report of an IVET system for use in the family *Pasteurellaceae*, and also the first that utilizes the IVET technology to analyze a respiratory pathogen in its natural host.

LOCATION OF THE SLT-I TOXIN GENES AND IDENTIFICATION OF REGULATORY, REPLICATION AND LYSIS GENES OF COLIPHAGE H-19B. Melody N. Neely and David I. Friedman. Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109.

Many virulence factors associated with bacterial pathogenesis are carried on genetic elements that facilitate their horizontal transfer amongst bacteria of the same or even different species. Bacterial viruses represent one class of natural vectors capable of transferring virulence determinants. We have been characterizing coli-lambdoid-phage H-19B, which carries genes encoding the Shiga-like toxin (SLT-I). Following infection, the H-19B genome, with its toxin genes, can integrate into the bacterial chromosome as a prophage, in this way transferring *slt* genes to a new host. Such *E. coli* derivatives expressing high levels of SLT have been isolated from stool samples of patients with acute bloody diarrhea and are referred to as enterohemorrhagic *E. coli* or EHEC. Infections with EHECs have been associated with contaminated food and their increasing worldwide occurrence highlights the danger they present to public health.

Analysis of the DNA sequence of a 15 kb region of H-19B located the genes encoding SLT-I downstream of the gene encoding the analog of the λ Q transcription activator with its site of action, the associated $p_R'$ late promoter, and upstream of the analogs of the λ genes encoding lysis functions. The orf corresponding to the holin lysis genes of lambdoid phages differs by having only one instead of the usual two closely-spaced translation initiation signals thought to contribute to the time of lysis. These observations suggest that *slt-I* expression is enhanced by transcription from $p_R'$ as well as a model for toxin release through cell lysis mediated by action of the phage-encoded lysis functions. We also find that H-19B shares a number of genes with a variety of lambdoid phages, although the repressor and operators are unique. The replication genes, except for a short in-frame insertion in the analog of the λ *O* gene that adds two extra iterons to the origin of replication, are essentially identical to the *O* and *P* genes of λ. We identify orfs and sequences in H-19B that resemble N transcription antitermination systems found in early operons of other lambdoid phages. We show that this N-like system fosters transcription antitermination, although it has significant differences from those of other lambdoid phages.

**An Elisa Assay To Detect Immune Responses To Antigens Using Biotin-Tagged Proteins: Evaluation Of Human Serum Antibody Response To *Shigella* Virulence - Associated Proteins.**

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Biotinylated hybrid proteins have been generated using gene fusions to several pathogenic determinants of Shigella. These include IpaA, IpaB, IpaC, IpaD, IpaH, VirG, ShET2 and Stx. Confirmation of constructs was done by Western blot analysis using monoclonal and peptide antibodies and alkaline phosphatase tagged avidin. By taking advantage of the high affinity between biotin and avidin, extracts from strains expressing individual hybrid proteins were plated on avidin coated microtiter plates. These were used in conjunction with human sera to perform ELISA assays generating immune responses to specific *Shigella* antigens.

Sera from two groups of volunteers were used to perform the assay. The first sera collection was tested for responses to IpaB, IpaH and ShET2 and was obtained from U.S. volunteers orally vaccinated with either placebo or SC602, an experimental live, attenuated, *Shigella flexneri* 2a vaccine. The individuals were subsequently challenged with a virulent *S. flexneri* 2a strain. The second sera collection, which was tested for antibodies to IpaB, IpaH, VirG and Shiga toxin, was obtained from volunteers living in an endemic region of Western Kenya during an outbreak of shigellosis in 1995, primarily due to *S. dysenteriae* 1. Initial results indicate that high titers to IpaB are uniformly present in the Western Kenya population. An evaluation of serum titers to individual *Shigella* antigens obtained with the two sets of sera will be presented. This assay demonstrates a simple approach for measuring immune responses to any protein to which biotin fusions can be constructed.

CHARACTERIZATION OF THE *esp*B HOMOLOG OF *CITROBACTER RODENTIUM* THE CAUSITIVE AGENT OF MURINE TRANSMISSABLE COLONIC HYPERPLASIA.

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*Citrobacter rodentium* is the causitive agent of transmissable murine colonic hyperplasia and shares virulence homology with enteropathogenic *Escherichia coli*(EPEC). The *esp*B gene has been shown to be necessary for intimate attachment and signal transduction between EPEC and host enterocytes. Mice challenged with wild-type *C. rodentium* develop a mucosal immunoglobulin A response to EspB.

The *esp*B gene homolog of *C. rodentium* has been cloned, and its nucleotide sequence has been determined to share 90% identity with the EPEC gene. The extent of virulence factor homology between the two organisms has been expanded to include the EPEC *esp*A and *esp*D genes, which play roles in transducing signals to epithelial cells. Homology has also identified between the predicted polypeptide of a 222bp ORF located downstream of the *esp*B gene homologue and the PrgI protein of *Salmonella typhimurium*, a protein involved in secretion of cell signalling proteins. Construction of a nonpolar insertional *esp*B mutant has been been constructed.

Results of the effects an *esp*B mutation have upon epithelial cell adherence and the induction of colonic hyperplasia will be presented.

# Expression Of *Salmonella typhimurium* Plasmid Encoded Fimbriae (Pef) Is Induced By Low pH.

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The *pef* operon (plasmid encoded fimbriae) is located on the virulence plasmid of *Salmonella typhimurium*. This locus facilitates the adherence of bacteria to the mouse intestine resulting in fluid accumulation. The *pef* operon is similar to the *pap* operon in *E. coli*, which is under DNA methylation dependent transcriptional control. The *pef* and *pap* operons share conserved upstream regulatory sequences as well as encoded proteins with significant homologies. These data suggest that the *pef* operon may be the first example of a DNA methylation regulated fimbrial operon outside *E. coli*.

Recently, conditions permitting the expression of Pef fimbriae have been identified. *S. typhimurium* express Pef fimbriae when grown in standing culture at 37°C in rich media (L broth) pH 5.1, buffered with 100 mM MES. Under these conditions 5% of the population immunostain positive for Pef fimbriae. This is a 30 fold increase in Pef fimbrial expression compared to *S. typhimurium* grown in unbuffered LB. This increase in fimbrial expression was confirmed by Northern blot analysis. Growth in LB pH 5.1, resulted in a 33 fold increase in *pefA* transcript level compared with growth in unbuffered LB. The transcriptional regulators of the *pef* operon *pefB* and *pefI* were also induced during culture in LB pH 5.1.

Genes involved in the regulation of the *pap* operon affect *pef* regulation as well. Mutations in *lrp* and *dam* reduce expression of Pef fimbriae. In contrast, mutant alleles of *rpoS* and *hns* resulted in increased numbers of fimbriated *S. typhimurium* during growth in LB pH 5.1 and unbuffered LB. These data indicate that both positive (*lrp* and *dam*) and negative (*rpoS* and *hns*) regulators as well as the environment modulate the expression of Pef fimbriae.

# HYPER-RESISTANCE TO INFECTION IN *Timp-1*-DEFICIENT MICE

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Timp-1 (tissue inhibitor of metalloproteinases) is one of a four-membered family of inhibitors of the extracellular matrix-degrading, matrix metalloproteinases (MMPs). Timp-1-deficient mice showed a dramatic resistance to corneal infection by *Pseudomonas aeruginosa.* During the first 4 hours of infection when bacterial attachment is occurring, and the next 8 hours when bacteria undergo an initial burst of replication, no differences were observed between mutant and wild-type mice. However, by 24 hours post infection at the peak of neutrophil infiltration, there were 500-1,000 fold fewer bacteria in the eyes of mutant mice than in eyes of wild-type animals. Administration of a synthetic MMP inhibitor, BB-94, suppressed 99.5% of the *Timp-1* mutant phenotype, demonstrating that the phenotype resulted from loss of MMP inhibitory activity. Depletion of the complement system from mice using cobra venom factor suppressed 95% of the mutant phenotype, demonstrating that enhanced bacterial resistance in the mutants was complement-dependent. This reveals that Timp-1 is a potent regulator of bacterial immunity, and that it operates through a complement-mediated mechanism.

A second, possibly related phenotype, was that the kinetics of neutrophil margination *in vivo* were elevated in *Timp-1* mutants. The enhanced capacity to mobilize neutrophils may also contribute to the enhanced infection resistance phenotype.

These results demonstrate that *in vivo,* complement-mediated antibacterial activity is extremely sensitive to Timp-1. Furthermore, it is likely that this sensitivity is mediated by an MMP that can participate in activating complement function. These studies are the first to demonstrate *in vivo* the link between a regulator of extracellular matrix remodeling and complement-based immunity.

## STUDY OF LIGAND-INDUCED MOLECULAR MOVEMENTS IN THE ASPARTATE RECEPTOR OF *S. TYPHIMURIUM*

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The mechanism by which ligands create a transmembrane signal in membrane receptor proteins has been studied using localized mutagenesis and spin-label probes in the aspartate receptor (Tar) of *Salmonella typhimurium* We have undertaken investigations to define molecular movements that occur upon ligand binding and creation of a transmembrane signal. In order to accomplish this, cysteine residues, either singly or in pairs, have been placed at different positions in the receptor by site-directed mutagenesis. These cysteine residues are chemically reactive, and act as sites that can be used for "marking" the receptor. These mutant receptors were then purified and reacted with the cysteine-reactive nitroxide spin label methanethiosulphonate (MTSSL). The electron paramagnetic resonance spectra of these labeled proteins were obtained in the presence and absence of ligand, and these spectra were used to calculate the distance between spin probes. This cysteine-cysteine distance can thus be evaluated for changes upon ligand binding. This method has been used to probe several sites in the protein, including in the periplasmic, linker and methylation domains. Although studies have demonstrated that there are regions of the receptor that undergo ligand-induced movement, it is clear that there are other regions that do not undergo significant changes upon ligand binding.

# SIGNAL TRANSDUCTION MECHANISMS AFTER *SALMONELLA TYPHIMURIUM* INFECTION

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*Salmonellae* represent an actual health problem being able to elude primary defense mechanisms like killing by professional phagocytes. To accomplish this the pathogens employ a number of different strategies. A common feature of these tactics is the interference with host's signal transduction mechanisms, aimed either at exploiting existing networks for the benefit of the pathogen or at interfering with key host function to delay the development of an antimicrobial response.

*Salmonellae* can survive and even multiply within macrophages escaping very hostile environment. In addition *Salmonella typhimurium* cultured to specific growth condition causes apoptosis of macrophages *via* a mechanism requiring a functional type III secretion machinery.

Our aim is to study the molecular mechanisms operating during interaction of the facultative intracellular pathogen *Salmonella typhimurium* with macrophages.

We show that at least two distinct mitogen activated protein (MAP) kinases pathways are activated upon *Salmonella* infection of macrophages: i. stress activated protein kinases (SAPK/JNK) and ii. extracellular signal regulated protein kinases (ERK). In our system SAPK/JNK activation occurs very rapidly, with the kinetics specific for *Salmonella typhimurium* infection.

In cell lines expressing activated raf the SAPK/JNK pathway is constitutively activated, while MAPK ERK1 and ERK2 activation is suppressed. Interestingly, these cells fail to undergo apoptosis upon *Salmonella typhimurium* infection, suggesting that either Raf or SAPK/JNK activation may in this system exert a protective effect against *Salmonella* infection.

A SINGLE GENE PRODUCT OF *RICKETTSIA TYPHI* PERMITS INVASION OF VERO CELLS BY *E. COLI*. S. Radulovic, J. M. Troyer, B. H. Noden, S. G. E. Andersson and A. F. Azad, Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD and Department of Molecular Biology, Uppsala University.

Entry into the eukaroytic cells is an essential step in the life cycle of *Rickettsia typhi*, the causative agent of murine typhus. Rickettsiae replicate intracellularly accumulating large numbers of progeny resulting in cell lysis and release. The molecular events permiting rickettsial entry into host cells have not been defined. *R. typhi* adhere to and lyse erythrocytes and , therefore we postulate a role for hemolysin-like substances in invasion of nonphagocytic cells. Here we describe the cloning and nucleotide sequence of the gene encoding putative hemolysin from *R. typhi*. We cloned this gene (1.3 kb) into expression vector pGEX-2TK (Pharmacia). Complementation experiments using *R. typhi tlyC* in a hemolysin deficient mutant *E. coli clyA*$^-$ were successful. *E. coli* expressing *R. typhi tlyC* (*clyA*$^-$ *tlyC*$^+$) invaded Vero cells and were able to replicate intracellularly. We have demonstrated that the cloned *R. typhi tlyC* was expressed and thereby confered the hemolytic phenotype onto otherwise nonhemolytic *E. coli* K-12. SDS/PAGE analysis of *E. coli clyA*$^-$ *tlyC*$^+$ revealed a band between 30-40 kDa which was not present in the *E. coli* carrying only the vector (*clyA*$^-$ *tlyC*$^-$). Using competition assays we observed that Vero cells infected with *E. coli clyA*$^-$ *tlyC*$^+$ inhibited entry of *R. typhi*, but not *R. akari* a member of SFG rickettsiae. The inability of *R. typhi* to enter into the infected Vero cells indicates a requirement to bind to host-cell receptor that have been used by mutant *E. coli*. Whereas the entry of *R. akari*, which lacks *tlyC* gene, into infected Vero cells was not inhibited indicating the utilization of different receptor molecules by this rickettsiae.

# A NOVEL LOCUS OF *SALMONELLA TYPHIMURIUM* INVOLVED IN PEYER'S PATCH SURVIVAL

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*Salmonella spp.* are responsible for disease symptoms ranging from self-limiting gastroenteritis to enteric fever. *Salmonella typhimurium* causes a typhoid-like condition in BALB/c mice, which can be used as a model for the human infection caused by *Salmonella typhi*. Studies from pathogenic bacteria suggest that those genes which are required for the infection process are usually regulated such that they are only expressed at the proper time and place during the infection process. The IVET technique (*in vivo* expression technology) was designed to identify operons which are transcriptionally active only when the bacterium is in the host. We have used this system to identify novel virulence genes of *Salmonella typhimurium*. required at various stages of the infection process. In particular, we have identified a novel locus of *Salmonella typhimurium* which is involved in survival or growth in the small intestine, the first step in the infection process. Fusions to this locus are transcriptionally active only during the early stages of infection in the small intestine, and are not expressed during systemic infection.. A null mutation was constructed and examined for attenuation after oral and intraperitoneal inoculation. This null mutation increases the $LD_{50}$ 5-fold following oral inoculation, but does not affect the $LD_{50}$ following intraperitoneal inoculation. Further studies have shown this mutant to be defective in survival in the peyer's patches. Sequence analysis indicates that this locus consists of a single open reading frame of approximately 1200 bp located on a cryptic lambdoid phage. It encodes a transposase-like protein that has homologs in a wide variety of organisms, ranging from Anabaena to Sulfolobus. In two organisms, *Dichelobacter nodosus* and *Helicobacter pylori*, homologs of this gene are associated with virulence determinants. Future studies will include determining the regulation of the operon both *in vitro* and *in vivo*, and to further characterize the mode of action of the gene product within the peyer's patch.

SEPTICEMIA PRODUCING *VIBRIO VULNIFICUS* HAS A HOMOLOG OF THE *VIBRIO CHOLERAE* TRANSMEMBRANE TRANSCRIPTION ACTIVATOR TOXRS

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*Vibrio vulnificus,* an estuarine bacterium, causes a fatal septicemia in susceptible subjects after ingestion of raw seafoods. In an attempt to dissect the virulence regulatory system in the bacterium, we examined the chromosomal DNA of *V. vulnificus* ATCC 29307 for sequences homologous to those of *V. cholerae toxRS* (Vc-*toxRS*)genes. By comparing the sequences of toxRS of *V. cholerae* and *V. parahaemolyticus* (Vp), we designed a set of degenerate primers targeting well-conserved sequences. The PCR product of 864 bp encompassing parts of both putative *toxR* and *toxS* was cloned, mapped, and sequenced. After confirmed of homologies with Vc-*toxRS* and Vp-*toxRS,* the cloned insert was used as the authentic probe. *V. vulnificus toxRS* (Vv-*toxRS*) could be localized to a 4.0-kbp *Bgl*II fragment by Southern hybridization of the chromosomal DNA. The whole 4.0-kbp fragment appeared to be lethal to *E. coli* competent cells. Based on the information from Southern hybridization and restriction mapping of the PCR product, the 4.0-kbp *Bgl*II fragment was cleaved into parts by *Hin*dIII. A 1.6-kbp *Bgl*II-*Hin*dIII and a 1.2-kbp *Hin*dIII fragment were finally cloned and sequenced. They contained two open reading frames attributable to Vv-*toxR* and Vv-*toxS.* The intact Vv-*toxRS* fragment in the chromosome was identified and cloned by PCR. The Vv-*toxR* shared sequence homology of 55% and 62% with Vc-*toxR* and Vp-*toxR,* respectively. Vv-*toxS* was 65% and 66% homologous to Vc-*toxS* and Vp- *toxS,* respectively. The deduced amino acid sequences of the Vv-*toxRS* gene product (ToxRS) showed regions similar to the proposed transmembrane and activity domains of Vc-ToxRS and Vp-ToxRS. Expression of Vv-ToxR and Vv-ToxS was observed as the GST fusion proteins. All clinical and environmental *V. vulnificus* strains examined proved to possess Vv-*toxRS* genes by DNA-colony blot hybridization. Other *Vibrio* species than *V. cholerae* or *V. parahaemolyticus* showed weak positive signals at low stringency. Vv-ToxRS promoted, in an *E. coli* background, the expression of *V. vulnificus* hemolysin gene by *ca* 5-fold. Vv-ToxRS also activated Vc-ToxR-regulated promoter incorporated in an *E. coli* (VM2) chromosome.

Pathways that present antigens from intracellular and extracellular pathogens on MHC class I molecules. Kenneth L. Rock*, Abie Craiu*, Zhenhai Shen*, Alfred L. Goldberg†, Tatos Akopian†, Maria Gaczynska†, and Gabriel Fenteany§. *University of Massachusetts Medical Center, †Harvard Medical School, §Harvard University.

MHC class I molecules in all cells bind and display to the immune system peptides derived from cellular and viral proteins. This allows the immune system to screen the expressed genes in tissues and destroy cells that are synthesizing foreign (e.g. viral) proteins. The majority of presented peptides are generated by degradation of proteins in the cytoplasm. We find that inhibitors of the proteasome block the degradation of the bulk of cellular proteins and inhibit the generation of the majority of peptides presented on MHC class I molecules. The peptides that fit into the antigen binding groove of class I molecules are 8-9 residues. We demonstrate in intact cells that cleavage by the proteasome defines the proper C-terminus of SIINFEKL from the antigen ovalbumin. However, a distinct peptidase(s) in the cytosol or endoplasmic reticulum generates the appropriate N-terminus of this epitope. Therefore, there are two distinct proteolytic steps in the generation of an antigenic peptide.

Dendritic cells and macrophages can also present on class I molecules antigens from the extracellular fluids. This pathway appears to be important for generating CD8 T cell immunity to pathogens that reside in phagosomes and possibly also to viruses that do not infect professional antigen presenting cells. The cell biology of this process is being characterized. Antigens are transferred from the endocytic compartment to the cytoplasm where they follow a final common pathway with other class I-presented proteins. Methods have been developed to efficiently target antigens into this pathway in vivo. Using this methodology protein-based vaccines can elicit both CD4 and CD8 T cell immunity.

Analysis of the Regulation of Mucoidy in Pseudomonas aeruginosa Infecting CYSTIC fibrosis patients using the Yeast Two hybrid SYSTEM

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Chronic respiratory infections with mucoid Pseudomonas aeruginosa mutants contribute signficantly to the high morbidity and mortality in cystic fibrosis (CF). The mucoid phenotype of P. aeruginosa, caused by the overproduction of the exopolysaccharide alginate, enables P. aeruginosa to resist pulmonary clearance and persist in the lungs of CF patients. The production of alginate is primarily controlled by the algU mucABCD gene cluster. Similar gene clusters have been found in other bacteria and are involved in the cellular response to extreme environmental stress. AlgU is an alternative sigma factor that is required for the expression of the alginate biosynthetic enzymes, while MucA is an anti-sigma factor for AlgU. MucB is another negative regulator of AlgU located in the periplasm. Genetic experiments have revealed that MucC also plays a regulatory role. It probably modulates MucA activity along with MucB as inactivation of mucC has no effects on mucoidy unless combined with a mucA or mucB mutation. To verify the presumptive interactions between these factors, we employed the yeast two hybrid system. A strong interaction was detected between MucA and AlgU, and a weak interaction between MucA and MucB was observed. No interaction was detected between MucA and itself. MucC was also cloned for use with the two hybrid system to test its presumptive interactions with MucA and MucB.

The most frequent site for mutations that cause mucoidy in P. aeruginosa isolates from CF patients is the mucA gene. The MucA protein contains a single putative transmembrane domain with the amino terminus thought to be in the cytoplasm and the C-terminus in the periplasm. Interestingly, a recent examination of 53 clinical isolates with mutations in mucA has revealed that most mutations cause a truncation of the C-terminal periplasmic domain of the MucA protein. We hypothesize that the mutations eliminate residues involved in the interaction of MucA with MucB or MucC, and that their association under non-stress conditions helps MucA adopt a confirmation that binds and inhibits AlgU. We cloned several representative mutant alleles of mucA from clinical isolates to study their ability to interact with AlgU, MucB, and MucC in the two hybrid system. A model of regulatory interactions was derived based on these analyses and additional studies in P. aeruginosa.

IDENTIFICATION OF GENES FROM AN EXTRAINTESTINAL ISOLATE OF *E. COLI* (CP9) WITH INCREASED EXPRESSION IN HUMAN URINE. T.A. Russo, U.B. Carlino, SUNY at Buffalo

Extraintestinal isolates of *E. coli* cause the majority of urinary tract infections (UTI) and this site is the source of 50-70% of *E. coli* bacteremias. Despite effective antimicrobial therapy, these infections cause considerable morbidity and mortality, resulting in a significant medical-economic burden to our national health care system. By understanding which bacterial determinants are important in the pathogenesis of UTI, we will be able to logically develop novel, effective strategies for the prevention or treatment of disease. To identify bacterial factors that have increased expression in the urinary tract, libraries of active Tn*phoA* and Tn*phoA'1* fusions were generated in a clinical isolate (CP9) and screened in human urine and L-B medium *in vitro*. Five genes have been identified that have increased expression in urine relative to L-B medium. 1) CP9.274 is an established isogenic derivative and possesses an active Tn*phoA* fusion in *artJ* (97% nucleotide homology with K-12 homologue) which is part of the periplasmic arginine transport system. Expression was increased 8.5-39.3-fold (median 23.6) in 4 independent urines evaluated. 2) CPI-2 is an established isogenic derivative and possesses an active Tn*lacZ* gene fusion in a divergent homologue of *betB* (36% identities/54% similarities). This gene encodes a betaine aldehyde dehydrogenase and is part of the pathway which converts choline to betaine, a critical osmoprotectant. Expression was increased 6.1-205-fold (median 55.6) in 15 independent urines evaluated. 3) CP9.45 is an established isogenic derivative and possesses an active Tn*phoA* fusion in a novel gene that appears to be part of a pathogenicity island. BLAST analysis of the 1.5kb 5' to the transposon insertion did not identify any gene or protein homologues, nor homologous K-12 sequence. Expression of this fusion is increased 2.4-75.4-fold (median 27.5) in 17 independent urines evaluated. Southern analysis was performed under high stringency conditions to determine the distribution of this gene in clinical *E. coli* isolates. 12/15 (80%) of UTI strains and 6/10 (60%) of bacteremic isolates were positive. 4) CP9.82 is an established isogenic derivative and has an active Tn*phoA* fusion in a divergent homologue of *fepA* (64% nucleotide homology with the K-12 homologue) which encodes the ferrienterochelin receptor. Expression was increased 3.2-131-fold (median 21.8) in 18 independent urines evaluated. 5) CP9.80 is an established isogenic derivative and possesses an active Tn*phoA* fusion whose expression is increased 9.3-60.2-fold (median 21.8) in 4 independent urines evaluated. The gene in which TnphoA has inserted has not yet been identified. Further evaluations of these strains *in vitro* and *in vivo* will clarify the roles of these newly identified genes in the pathogenesis of UTI.

# MEMBRANE FATTY ACID COMPOSITION MODULATES *SHIGELLA FLEXNERI* VIRULENCE

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In *Shigella flexneri* the invasion genes ipa are temperature-regulated since they are activated in bacteria grown at 37°C but not at 30°C. VirB is the positive regulator which is in turn positively regulated by VirF and negatively by the histone-like protein H-NS according to temperature. We investigated whether, in *Shigella flexneri* membrane lipid composition, and particularly the ratio of saturated to unsaturated fatty acids, was involved in the perception of external stimuli such as temperature. To test this hypothesis either fatty acids or ethanol were added to *shigellae* growing at 37°C and several parameters associated to virulence, including fluidity of membranes, were analyzed. The addition of palmitic acid lowers both invasiveness and the IpaB and IpaC production. Analysis of both β-galactosidase activity of the transcriptional fusions ipaB::lacZ and ipaA-lacZ, and the amount of ipa mRNA transcript revealed that ipa transcription was significantly decreased. Conversely, when an hns::Tn10 mutation was introduced into either M90T or M90T ipaB::lacZ or M90T ipaA-lacZ the negative effect of palmitic acid was relieved and the levels of both ipa transcription and invasiveness were comparable to those of bacteria cultured in unsupplemented medium. Trans-complementation of the hns::Tn10 mutants with a plasmid carrying the entire hns gene restored the repression of ipa transcription induced by the presence of palmitic acid.
This result strongly indicates that palmitic acid addition may modulate the virulence of *shigellae* at the transcriptional level through H-NS . Moreover, ethanol, that perturbs membrane composition by enhancing the incorporation of unsaturated fatty acids, represses the expression of ipa operon and the virulence.

EFFECT OF HETEROLOGOUS EXPRESSION OF *Salmonella typhimurium* OmpD PORIN IN *Salmonella typhi*: ENTRY INTO AND PROLIFERATION WITHIN CULTURE CELLS AND VIRULENCE IN MICE.

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*S. typhi* is the only *Salmonella* serovar that grows exclusively in humans causing typhoid fever. On the other hand, *S. typhimurium* produces a typhoid-like disease in mice, being less pathogenic for humans. The lack of OmpD porin is the major difference between *S. typhi* and *S. typhimurium* outer membrane (OM) proteins. Although OmpD is present only in the *S. typhimurium* OM, *ompD*::Tn*10* transduction from *S. typhimurium* into *S. typhi* chromosome suggests the presence of the homologous gene in the latter. Therefore, it is posible that *ompD* is a silent gene in *S. typhi*.

We transduced, via P22 phage, the *S. typhimurium ompD* gene into *S. typhi* and studied its expression in the *S. typhi* genetic background. Under acidic culture conditions (pH 5.0), the porin is repressed. It is induced under anaerobic growth conditions in a Fnr-dependent manner, as seen using *fnr*::Tn*10* mutants. Furthermore, no regulation is detectable by medium osmolarity, accordingly to the observation that an *envZ* null mutation does not affect its expression. These regulations are also observed for *ompD* in *S. typhimurium* .

To evaluate whether OmpD has some rol in host specificity, we have tested our strains to asses their ability to entry into and proliferate within J774 cells, a murine derived macrophage-like cell line. Our results show that *S. typhi* OmpD$^+$ penetrates J774 cells as efficiently as the wild type strain, however, its intracellular proliferation is four-fold higher. These results indicate that the heterologous OmpD is somehow enhancing *S. typhi* intramacrophage survival. In addition, we have assayed virulence by intraperitoneal injection of bacteria into BALB/c mice. Using a *S. typhimurium* virulent strain, we observed that the lack of OmpD porin does not modify the median lethal dose ($LD_{50}$) obtained with the wild type parental strain. When *S. typhi* strains were assayed, it was necessary to inject bacteria in mucin suspension. Fourteen days after inoculation, *S. typhi* OmpD$^+$ showed the highest viable count recovered from spleen. Again, this result supports that *S. typhi* survival in mice has been enhanced by OmpD porin presence. The question whether *ompD* is present in *S. typhi* still remains.

THE RAPID UPREGULATION OF INTERLEUKIN-10 IN CYTOTOXIC PSEUDOMONAS AERUGINOSA PNEUMONIA.

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*Pseudomonas aeruginosa* is now the most frequent Gram-negative bacteria associated with lung infection in critically ill patients or cystic fibrosis patients. Despite potent new antibiotics, the mortality of this pneumonia is still high. We have characterized the virulence of various *P.aeruginosa* strains (wild-type: PA103, PA01, and genetically modified strain: PA103 tox::Ω, PA103 exsA::Ω) when administered into the lungs of mice. Only strains (PA103, PA103 tox::Ω) which have the ability to produce exoenzyme S are highly cytotoxic, causing 100 % mortality within 48 hours at a dose of $5 \times 10^5$ CFU in this mice model.

To investigate the host response after administration of these cytotoxic strains, the mRNA levels of several cytokines in the mice lung homogenates were measured using RT-PCR. All bacterial strains caused an increase of the major proinflammatory cytokines such as IL-1β, IL-6, TNF-α in the mice. However, a significant increase of IL-10 mRNA was detected only in the mice who had received cytotoxic *P.aeruginosa* strains (PA103 and PA103 tox::Ω). The rapid increase of IL-10 mRNA was confirmed in three different inbred mouse strain (BALB/c, DBA, C57BL/6) and in SCID mice infected with cytotoxic *P.aeruginosa* strains. Protein ELISA further confirmed the increased level of IL-10 in blood samples of these mice. We found that the main source of this IL-10 was alveolar macrophages. The IL-10 upregulation was also observed in macrophage like cell lines (J774A.1) exposed to the cytotoxic *P.aeruginosa* strains. The rapid upregulation of IL-10 gene might have some key roles in early process of *P.aeruginosa* infection.

PHOSPHOLIPASE A OF *YERSINIA ENTEROCOLITICA* CONTRIBUTES TO PATHOGENESIS IN A MOUSE MODEL.

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*Yersinia enterocolitica* causes a lymphotrophic infection in humans. The same progression of infection is seen in peroral infected mice. Previous work by Aulisio et al., (1983) correlated lipase activity on egg yolk agar with virulence of *Y. enterocolitica* strains in mice by peroral $LD_{50}$, intraperitoneal $LD_{50}$, and Sereny test. Thus we have isolated a *Y. enterocolitica* cosmid positive for lipase activity in *E. coli* (on Tween 80 agar plates). After subcloning a 6 kb fragment into pUC19, the region required for lipase activity was identified by transposon mutagenesis. Approximately 2 kb encompassing this region has been sequenced, and two open reading frames (orf) were found. One orf has 74% identity and 85% similarity to the phospholipase A found in *Serratia liquifaciens*. Though the other orf was less similar to the downstream accessory protein found in *S. liquifaciens*, the organization in both species is similar. Furthermore, crude extracts of *Y. enterocolitica* were found to have phospholipase A activity by a spectrophoto-metric assay. In addition, when egg yolk lecithin (phosphatidylcholine) was substituted for Tween 80 in plates, *Y. enterocolitica* produced a large halo of precipitated fatty acid. Subsequently, a phospholipase negative *Y. enterocolitica* strain was constructed by disrupting the chromosomal copy of the gene with a suicide plasmid. The resulting strain was negative both on the Tween 80 and lecithin plates and in the spectrophotometric assay. This supports the conclusion that a *Y. enterocolitica* phospholipase A gene has been isolated. To ascertain whether the phospholipase has a role in pathogenesis, the mutant strain was tested in the mouse model. Experiments using peroral infected BALB/c mice has repeatedly shown the mutant gives significantly less recoverable viable counts from the mesenteric lymph nodes than the parental strain 3 or 5 days post infection. Furthermore, bowel tissue and Peyer's patches infected with the mutant strain appear to be less inflamed than those infected with the parental strain. Therefore, tissue samples were sectioned, stained and examined for inflammation and necrosis. Mouse tissues infected with the mutant appear to have less severe or fewer foci of inflammation and/or necrosis. This results is not surprising if fewer viable bacteria were recovered. However, at high infectious doses similar numbers of viable parental and phospholipase negative mutants were recovered from the Peyer's patches and mesenteric lymph nodes, yet the numbers of foci and extent of inflammation and necrosis were noticeably less in the tissues infected with the mutant than the parental strain. Together these findings suggest that *Y. enterocolitica* produces a phospholipase A which has a role in pathogenesis. Whether the phospholipase is important in yersiniae infections for carbon acquisition, tissue disruption via membrane degradation, or modulating the immune response by producing the inflammatory mediator arachidonic acid, has yet to be determined.

COORDINATE REGULATION OF VIRULENCE FACTORS OF *CAMPYLOBACTER* SPP. BY BILE SALTS.

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Bile salts, including deoxycholate (DOC), have previously been shown to induce the formation of pilus-like structures on *Campylobacter jejuni* and *Campylobacter coli* cells. Loss of the ability to express these pilus-like structures in *C. jejuni* 81-176 reduced virulence in a ferret diarrheal model, suggesting that the structures play a role in pathogenesis. The effect of growth in DOC on other virulence factors of *Campylobacter* spp. was examined. Incorporation of DOC into motility agar reduced motility of *C. jejuni* and *C. coli* . Primer extension analyses indicated that transcription of the $\sigma^{28}$ controlled *flaA* gene, encoding the major flagellin in the complex flagellar filament, was repressed in DOC grown cells. A fusion of the $\sigma^{54}$ regulated *flaB* gene to a promoterless chloramphenicol acetyl transferase (CAT) gene was used to measure the effect of DOC on expression of the minor flagellin. These experiments indicated that expression of the *flaB* gene is enhanced by approximately four-fold in the presence of DOC. Growth in DOC also enhances expression of cytolethal distending toxin (CDT) by several strains of *C. jejuni* from 6 to 42 fold as measured by HeLa cell assays. Reverse transcriptase-PCR studies indicated that the enhanced CDT levels are due to an increase in the amount of a single mRNA encoded by the *cdtA*, *cdtB* and *cdtC* genes in DOC grown cells. Levels of invasion of *C. jejuni* 81-176 and *C. coli* VC167 T2 are also increased by approximately 3-fold when grown in 0.1% DOC. The data collectively suggest that expression of campylobacter virulence determinants are coordinately modulated by bile salts.

**THE SPI2 TYPE III SECRETION SYSTEM OF *SALMONELLA TYPHIMURIUM* IS REQUIRED FOR GROWTH IN THE MOUSE SPLEEN.**

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The *Salmonella typhimurium* genome encodes two type III secretion systems. One is present on a pathogenicity island (SPI1) located at 60 centisomes and plays an important role in epithelial cell entry. The other is also present on a pathogenicity island (SPI2) at 30.5 centisomes and is required for systemic infection.

To determine where and when the SPI2 mutant phenotype first becomes apparent, mice were inoculated singly with wild type (WT) or mutant strains, or with a mixture of strains, and the bacterial load of each strain was determined over time in various organs including mesenteric lymph nodes, liver and spleen. SPI2 mutants were present in similar numbers to WT strains in these organs up to 16 hours post inoculation. At 16 hours significantly more WT bacteria were recovered from the spleen. The numbers of SPI2 mutant bacteria remained relatively constant for 10 days post inoculation, while the numbers of WT bacteria increase rapidly until death of the animal at 3-4 days post inoculation. SPI2 mutant cells persist at gradually decreasing levels in both liver and spleen for a further 8 to 10 weeks.

To investigate the role of the SPI2 secretion system *in vivo*, measurements of intracellular bacteria within the spleen were made during the early stages of infection. A non-segregating plasmid was also used to measure the growth and killing of the SPI2 mutant strain in relation to the WT strain. Results of these experiments fail to support the hypothesis that the SPI2 secretion system is required for intracellular survival and replication. Alternative hypotheses for the function of SPI2 will be presented.

# INVESTIGATING PROTEIN-DNA AND PROTEIN-PROTEIN INTERACTIONS DURING *SALMONELLA* PLASMID VIRULENCE (*SPV*) GENE EXPRESSION.

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In *Salmonella typhimurium* and other nontyphoidal serovars of *Salmonella*, the plasmid-linked virulence locus *spv* is required for the induction of systemic disease in laboratory mice. The *spv* locus consists of four cotranscribed structural genes, *spvABCD* and the independently transcribed regulatory gene, *spvR*. The *spvR* gene product is a LysR-type transcriptional activator, required for the expression of both *spvR* and the *spvABCD* operon. Transcription of *spvR* and the four structural genes is induced during stationary phase *in vitro* and following the ingestion of salmonellae by macrophages and epithelial cells. This induction requires the alternative sigma factor RpoS.

Previous studies have shown that SpvR binds to the *spvA* and *spvR* promoters *in vitro*. We have further characterised the SpvR-DNA interaction using a sensitive footprinting assay for studying DNA-protein interactions *in vivo*. Our *in vivo* dimethyl sulfate footprinting experiments demonstrate that SpvR binds at a single site in both the *spvA* and *spvR* promoters. Our experiments suggest that SpvR makes intimate contact with nucleotides on one face of the DNA helix. Mutations within the ca. 20 bp contacted by SpvR decrease or abolish DNA binding *in vivo*, with concomitant effects on gene expression.

SpvR is the first regulatory protein to be described that interacts with RNA polymerase containing RpoS. We have probed for interactions between SpvR and RpoS. Preliminary evidence suggests that SpvR does not directly interact with RpoS. In contrast, our evidence suggests that SpvR forms dimers or higher multimeric structures and may activate virulence gene expression through contacts with the α-subunit of RNA polymerase.

## LIPOOLIGOSACCHARIDE INNER CORE BIOSYNTHESIS IN *NEISSERIA MENINGITIDIS*: ROLE OF A PHOSPHATASE HOMOLOG

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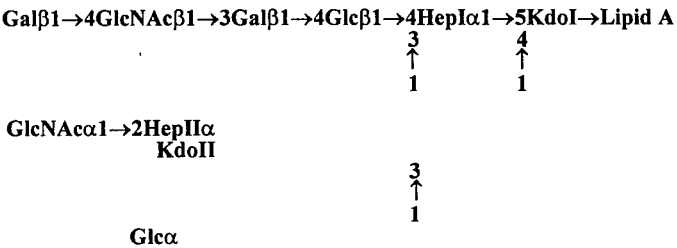
Lipooligosaccharide (LOS) is a critical virulence factor of *N. meningitidis*. To elucidate the genes involved in LOS biosynthesis, Tn*916* insertion mutants in a clinically isolated *N. meningitidis* serogroup B strain (designated NMB) [B:2b:P1.2,5:L2] were screened for loss of reactivity with monoclonal antibody 3F11, which specifically recognizes the unsialylated terminal galactose on the α-chain paragloboside of neisserial LOS. Tn*916* insertion mutant 469 was identified and found to exhibit a markedly truncated LOS of 2.9 kDa when compared to the parental LOS (4.6 kDa) on tricine SDS-PAGE. Composition analysis by electrospray mass spectrometry (ES-MS) and NMR spectroscopy revealed that the parent strain NMB displayed a L2 LOS structure:




Mutant 469, in contrast, exhibited a deep rough LOS phenotype consisting of $Kdo_2$→Lipid A. Sequencing of chromosomal DNA flanking the Tn*916* insertion in mutant 469 revealed that the transposon had inserted into an open reading frame (*orfA*) that was predicted to encode a 188 aa protein with homology (29% identity, 42% similarity over 139 aa) to *E. coli* HisB, a bifunctional enzyme with imidazole glycerol-phosphate dehydratase and histidinol-phosphate phosphatase activity. The homology between OrfA and HisB spanned the N-terminus of HisB, the region responsible for histidinol-phosphate phosphatase activity. Specific polar and non-polar *orfA* mutations in NMB reproduced the deep rough LOS phenotype of mutant 469. Analysis of the lipid A portion of NMB and 469 LOS by ES-MS revealed that NMB LOS lacked the C4′ phosphate on lipid A, whereas this phosphate was present on lipid A of 469 LOS. These results suggest that a C4′ phosphate is present on lipid A and then removed, possibly by the *orfA* gene product, prior to the addition of HepI and completion of the LOS molecule. Alternatively, the *orfA* gene product is required for removal of phosphate at another position on the $Kdo_2$→Lipid A structure.

FUNCTIONAL ANALYSIS OF THE GENE ENCODING cAMP RECEPTOR PROTEIN FROM *VIBRIO CHOLERAE*

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The cyclic AMP (cAMP) receptor protein (CRP) is a global regulator of gene expression in enteric bacteria and plays a role in the regulation of virulence gene expression and pathogenesis in *Vibrio cholerae*. As a first step toward elucidating the molecular mechanisms by which the cAMP-CRP system negatively regulates the coordinate expression of the genes encoding cholera toxin (*ctx*) and toxin coregulated pilus (TCP) in response to environmental stimuli, we have cloned the *V. cholerae crp* gene using a chromosomal capture method and determined its complete nucleotide sequence. The gene shows 81% identity with the *crp* gene from *Escherichia coli* and its deduced amino acid sequence shows 95% identity with the *E. coli* protein. When expressed under the control of the inducible promoters PAraBAD or Ptac, the *V. cholerae crp* gene produces a 23 kDa protein which complements the carbohydrate-negative and growth-defective phenotypes of *crp* mutants in both *E. coli* and *V. cholerae*. The gene also complements the *V. cholerae crp* mutant by restoring the normal repression of ToxR-regulated virulence genes under certain environmental conditions. The possibility that cAMP-CRP represses virulence gene expression by binding to a consensus sequence within the *tcpA* promoter is currently under investigation.

# CRYSTALLIZATION AND PRELIMINARY DIFFRACTION OF THE NON-CATALYTIC DOMAN OF YOPH FROM YERSINIA PSEUDOTUBERCULOSIS

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*Yersinia* infections cause diseases ranging from gastroenteritis to the bubonic plague. The ability of the bacteria to resist non-specific host defense is due to the presence of a virulence plasmid that encodes several secreted proteins, called *Yersinia* outermembrane proteins (Yops). Currently, seven of these proteins have been shown to be essential for virulence. When a bacterium makes intimate contact with a eukaryotic target cell, usually a macrophage, the extracellularly located bacterium secretes Yops by the type III secretion machinery, which is encoded by the virulence plasmid. Next, the effectors are translocated through the plasma membrane into the interior of the target cell. Secretion and translocation occur in a polarized manner that prevents secretion of the Yops in the surrounding medium, thus avoiding the primary host defenses.

YopH is one of the effector proteins that is encoded on the virulence plasmid and secreted by the type III secretion pathway. Its C-terminal domain encodes a protein tyrosine phosphatase that has been shown to cause dephosphorylation of target cell proteins and disrupt phosphotyrosine signaling associated with the bacterial uptake process. Recently, it has been shown that YopH targets $p130^{Cas}$, a protein important in focal adhesion assembly. The first 71 residues of the N-terminal domain contain regions that are responsible for directing the protein to the type III secretion machinery for secretion across the bacterial membrane and translocation across the eukaryotic membrane.

To gain a better understanding of how the secretion and translocation process works, we have crystallized the N-terminal domain of YopH from *Yersinia pseudotuberculosis*. Crystals diffract to at least 2.2 Å and belong to the orthorhombic space group C222 with unit cell parameters a=48.2 Å, b=121.7 Å, c=49.1 Å. Heavy atom screening is now in progress.

# ACONITASE AND THE ENVIRONMENTAL SIGNALS THAT CONTROL *PSEUDOMONAS AERUGINOSA* EXOTOXIN A PRODUCTION

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The expression of exotoxin A (PE) by *Pseudomonas aeruginosa* requires entry into stationary phase, a low iron concentration, and a complex media with high glutamate. To understand the requirement for glutamate, the amino acid composition of the medium was varied and PE production was examined using a *toxA* promoter *lacZ* fusion. It was found that a number of amino acids can substitute for glutamate, confirming previously published results. It was also found that citric acid cycle intermediates could also replace glutamate. From this information we concluded that the nitrogen content of the amino acid was irrelevant, and that the amino acid requirement might be due to a block in the citric acid cycle. As PE is produced only under conditions of low iron concentration, a likely place for the block in the citric acid cycle is at the conversion of citrate to isocitrate; a reaction catalyzed by aconitase an iron-sulfur center containing enzyme. In examining aconitase activity we found an inverse correlation between PE production and aconitase activity. Further confirmation of this conclusion was obtained from the observation that there was very little expression from the *toxA* promoter when aconitase was artificially stabilizing with the analog flourocitrate in cultures grown under toxigenic conditions. In summary, the inverse correlation between aconitase and PE synthesis suggests that the glutamate requirement stems from an iron limitation-dependent inactivation of aconitase. Furthermore, our results suggest the possibility that aconitase may directly regulate PE synthesis.

# MECHANISM OF HORIZONTAL GENE TRANSFER- THE VIBRIO CHOLEARE 0139 PARADIGM

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*Vibrio cholerae* serogroup 0139 Bengal is the only serogroup other than 01 known to cause epidemic cholera. It has been postulated that 0139 Bengal probably arose from an 01 strain. At the molecular level, it has been shown that the recipient strain had undergone a deletion of the approximately 22kb 01 *rfb* region and acquired a 35 kb region which encodes 0139 surface polysaccharide. Two mechanisms have been proposed to explain the origin and transfer of the novel DNA resulting in the new antigen. 1. Homologous recombination on either side of the 01 *rfb* genes resulting in a deletion/insertion of the *rfb* genes. 2. Insertion of the new O antigen DNA is due to an illegitimate recombination event mediated by an IS element such as IS *1358* present in many *V. cholerae* strains including the 01 and 0139 strains. In either case, neither the mode of transfer (conjugation or transduction) nor the source of the donor DNA is known.

To address the first hypothesis, we have investigated the left and right junctions as potential recombination sites. It has already been shown that the left junction gene *rfaD* is highly conserved among several *V. cholerae* strains. We have cloned and sequenced the right junction gene. The conserved nature of the right junction gene has been analyzed in several other *V. cholerae* strains.

To address the second hypothesis, we have undertaken a study to dissect the mechanism of transposition by IS *1358*. We have cloned a 1.4 kb DNA fragment containing IS *1358* from *V.cholerae* 0139 and marked the element with a *tetR* determinant. We have also cloned the IS *1358* transposase gene under T7 promoter and this clone over expresses a protein of 42 kd. A three plasmid system is being utilized to analyze the transposition process *in vivo*.

*SALMONELLA TYPHIMURIUM* CAUSE THE AGGREGATION OF PRE-FORMED HOST LYSOSOMES BY A MECHANISM INVOLVING *SIFA*.

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*Salmonella typhimurium* is a leading cause of gastroenteritis in humans and causes a systemic infection similar to typhoid fever in mice. This bacteria has the ability to enter into both phagocytic and non-phagocytic cell types and survival and replication within these intracellular compartments is a key virulence trait. During *Salmonella*'s interaction with a model system for non-phagocytic cells, HeLa epithelioid cells, a novel intracellular phenotype is observed. *Salmonella* trigger the apparent aggregation of lysosomal membrane glycoprotein-containing vesicles into tubular structures. These tubules interconnect with the *Salmonella*-containing vacuole and their formation coincides with intracellular replication.

At least one virulence-associated bacterial gene, *sifA*, is required for the formation of these structures by *S. typhimurium*. *sifA* encodes a 38 kD protein and the gene is located within a housekeeping operon and probably arose by horizontal transfer. Disruption of *sifA* also alters the rate of intracellular bacterial replication. The doubling time for a *sifA* mutant is 2/3 that of wild-type.

Evidence will be presented that indicate the tubules induced by intracellular *Salmonella* arise from aggregation of pre-formed lysosomes. Results showing that *sifA* is conserved among most *Salmonella* subspecies will also be presented. However, *sifA* is absent from at least one subspecies (IIIb) still able to induce lysosomal membrane glycoprotein-containing tubules within host cells. These results suggest that *Salmonella* specifically alter host cell endocytic trafficking via a mechanism that can involve *sifA* and probably requires other unidentified genes.

# DAMAGE BY ENDOGENOUS SUPEROXIDE IMPLIES THAT PHAGOCYTES ATTACK THE ACHILLES' HEEL OF BACTERIA

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Superoxide is a reactive oxygen species that is produced phagocytes defending against bacterial infection. Its toxicity is evidenced by the presence of superoxide dismutase (SOD), in all aerotolerant organisms. In fact, *Escherichia coli* contains a huge amount of SOD, approximately 3000 U/mL. It is conceivable that large amounts of SOD are made to protect against phagocytic attack. Mutants lacking cytosolic SOD exhibit severe growth defects due to inadvertent endogenous superoxide production associated with aerobic respiration. They display several auxotrophies and suffer enhanced DNA damage. Recent work has identified one target of superoxide in the cell; one type of [4Fe-4S] cluster found in some dehydratase enzymes. The oxidation of the clusters inactivates the enzymes, resulting in the auxotrophies, and liberates free iron which contributes to the DNA damage. However, it is not clear how much endogenous superoxide levels need be increased for these phenotypes to arise.

We constructed a strain in which the amount of cytosolic SOD made can be externally modulated. This allows us to vary steady-state concentration of superoxide. The specific activities of superoxide labile dehydratases, aconitase and 6-phosphogluconate dehydratase, drop to nearly 60% with only a 3-fold increase in superoxide. This enzyme inactivation results in higher levels of free iron that were detected by whole-cell EPR. In addition, oxidative DNA damage was shown to dramatically increase with 5-fold higher endogenous superoxide.

These results suggest cells have only enough SOD to protect themselves during normal growth. Superoxide produced in the acidic phagocyte is likely to be protonated and able to cross membranes. Thus, a small amount produced by the phagocyte would be very damaging to the bacterium. Peroxynitrite, also produced by phagocytes, similarly crosses the membrane and oxidizes the dehydratase clusters. Either could easily upset the fragile balance between enzymes and endogenous superoxide. Superoxide or peroxynitrite would cause enzyme damage and iron leakage which, in combination with $H_2O_2$, would be lethal to the invading bacterium. Thus, it appears that the phagocytic attack is focused on a existing vulnerability of the bacterium.

CHARACTERIZATION OF TYPE IV PEPTIDASE MUTANTS OF *AEROMONAS HYDROPHILA* AND *VIBRIO VULNIFICUS* MAY LEAD TO THE IDENTIFICATION OF NOVEL VIRULENCE DETERMINANTS

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Type IV leader peptidases/*N*-methyltransferases (herein referred to as Type IV peptidases) are bifunctional enzymes responsible for cleavage of the unique leader peptide and subsequent *N*-methylation of the newly exposed N-terminal amino acid of two classes of proteins required for pathogenesis of many Gram-negative bacteria. One class consists of proteins essential for biogenesis of Type IV pili, including the structural pilin subunit and several pilin-like proteins required for pilus assembly. The second group includes several proteins that comprise part of the machinery for extracellular secretion of proteins via the general secretory pathway (GSP or type 2 secretion). Studies undertaken to characterize the role(s) of the Type IV peptidase in expression of virulence determinants from *Aeromonas hydrophila* and *Vibrio vulnificus*, two significant pathogens of humans and living marine resources, have provided strong evidence for the existence of several as yet unidentified factors important for virulence of these species.

A mutation in the gene encoding the Type IV leader peptidase of *A. hydrophila* (*tapD*) is unable to process the precursor of a prototypical Type IV pilin (TapA); however, *tapA* and *tapD* mutants still express surface pili. When examined for the ability to adhere to HEp-2 cells, a *tapA* mutant shows no reduction in adherence while a *tapD* mutant is less adherent. While loss of TapD results in a pleiotropic protein export defect and the inability to secrete aerolysin and protease across the outer membrane, aerolysin mutants show no decrease in HEp-2 cell adherence. Therefore, we postulate that other cell surface or secreted proteins that follow the GSP are potential adherence factors in this pathogen.

A similar mutation in the Type IV leader peptidase gene (*vvpD*) of *V. vulnificus* results in elimination of surface pili and a defect in extracellular export of a hemolysin, the *V. vulnificus* cytolysin. The *vvpD* mutant shows a marked decrease in HEp-2 cell adherence and cytotoxicity. In addition, the *V. vulnificus vvpD* mutant is significantly less virulent in the iron-overloaded mouse model. Work from other laboratories has shown that elimination of the cytolysin alone does not affect virulence of *V. vulnificus* in mice, and until now, the only genetically engineered mutants with attenuated virulence are those deficient in polysaccharide capsule biosynthesis. In contrast, the *vvpD* mutant of *V. vulnificus* is still encapsulated and is resistant to the complement-mediated bactericidal effects of human serum. These results suggest that pili and/or one or more exported proteins whose expression and normal localization are under the control of VvpD are required for virulence of *V. vulnificus*.

THE (α1→6)-LINKED N-ACETYL-D-MANNOSAMINE-1-PHOSPHATE CAPSULE OF SEROGROUP A *NEISSERIA MENINGITIDIS* IS PRODUCED BY A NOVEL BIOSYNTHETIC GENE CASSETTE

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Epidemic serogroup A *Neisseria meningitidis* continues to pose a substantial threat to human health. The (α1→6)-linked N-acetyl-D-mannosamine-1-phosphate meningococcal capsule which determines serogroup A specificity is biochemically distinct from the capsules produced by other disease-associated meningococcal serogroups (B,C,Y,W-135) which contain sialic acid. We have defined the genetic cassette responsible for biosynthesis of the serogroup A capsule. Like the sialic acid biosynthetic gene cassettes, the serogroup A cassette was located between *ctrA*, encoding an ABC outer membrane transporter homologue and *galE*, encoding the UDP-glucose-4-epimerase. However, the genes of the serogroup A gene cassette were distinct from the sialic acid-producing meningococcal capsule biosynthetic gene cassettes. Four novel open reading frames (ORFs) were identified in the 4703 bp sequence located between *ctrA* and *galE* in serogroup A *N. meningitidis*. By PCR and Southern hybridization these genes were not present in meningococci of other major serogroups (B,C,Y,W-135). The four ORFs were separated from *ctrA* by a 218 bp intergenic region and were transcribed in the opposite orientation to *ctrA*. The first ORF exhibited 58% identity with a UDP-N-acetyl-D-glucosamine (UDP-GlcNac) epimerase in *E. coli*, responsible for converting UDP-GlcNac into UDP-N-acetyl-D-mannosamine. RT-PCR and primer extension studies of mRNA from the serogroup A strain showed that all four ORFs were co-transcribed and the start site of transcription was present within the 218 bp intergenic region. Transcription of *ctrA* was divergently initiated from within the intergenic region by a putative σ-70-type promoter that directly overlapped the putative σ-70 ORF1 promoter. Polar or nonpolar mutagenesis of the two upstream ORFs resulted in an abrogation of capsule production as assayed by colony immunoblots. These data suggest that the genes responsible for the biosynthesis of serogroup A capsule from monomeric N-acetyl-D-glucosamine are contained in a genetic cassette that distinguishes serogroup A meningococci. Accordingly, we have designated these four genes *sacA*, *sacB*, *sacC* and *sacD* (serogroup A capsule biosynthesis genes). The biochemically distinct capsules of *N. meningitidis* are the result of serogroup-specific biosynthesis gene cassettes located between *ctrA* and *galE* which can undergo recombinational exchange.

# INVASION OF HELA CELL BY *E. COLI* K-12 STRAIN USING RECOMBINANT IPA PROTEINS OF *SHIGELLA SONNEI*

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The ability of shigellae to enter the cytoplasm of intestinal epithelial cell is attributed to a large plasmid which contains genes encoding invasion plasmid antigens(Ipa), including IpaB,C,D proteins. The secretion of these proteins is increased when the bacteria have come to have a contact with the host cells. It has been shown that once secreted, IpaB-C complex of *Shigella flexneri* could promote entry of latex beads bearing these proteins into HeLa cells.

In order to reconstitute Ipa proteins complex as a functional effector molecule for invasion, we have expressed IpaB,C,D of *Shigella sonnei* as fusion proteins either with maltose binding protein or Strept-tag sequence. Affinity purified crude IpaB,D are separated from MBP by digestion with Factor *Xa*. Recombinant IpaC having Strept-tag sequence at its C-terminal was also purified by avidin affinity column. In gentamicin protection assay, these recombinant proteins showed the ability to cause non-invasive *E. coli* K-12 strain to internalize HeLa cell when all of the proteins were mixed. Electron microscopy also showed internalized bacteria within HeLa cells suggesting that functional complexes of invasins were formed *in vitro*. Since all three Ipa proteins were necessary to cause internalization of non-invasive *E. coli* into HeLa cell, IpaD might play a role as a anchoring molecule between the bacterium and IpaB-C complex.

ISOLATION OF A NOVEL CHROMOSOMAL LOCUS WHICH CONTAINS *BFPT*-REGULATED GENES IN ENTEROPATHOGENIC *E. COLI*

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The *bfpTVW* operon, also known as the *per* operon, of enteropathogenic *E. coli* (EPEC) is necessary for the transcriptional activation of the *bfp* operon, which encodes the major subunit and assembly machinery of bundle-forming pili (BFP). Both the *bfpTVW* and *bfp* operons are localized on the entero-adherent plasmid (EAF) of EPEC. *bfpT* is predicted to encode a 31.8-kD protein that shares homology with the AraC family of transcriptional regulators, including the presence of a conserved C-terminal DNA-binding helix-turn-helix motif. To purify the BfpT protein, a T7-tagged BfpT fusion protein was produced and immobilized on magnetic beads (Dynabeads). This immobilized BfpT protein reagent as used to show specific binding of BfpT to DNA from the upstream promoter region of *bfpA*. The immobilized BfpT protein reagent was also used to "fish out" from a promoter library, other EPEC chromosomal fragments that bind the BfpT protein. From this collection of BfpT binding promoter fragments, several were introduced into a pair of EPEC strains, one which harbors the *bfpTVW* clone and the other which does not. The promoter activities in each pair of transformants were then compared. Some of the promoters were found to be positively regulated by *bfpTVW*, whereas a few others were negatively regulated. One of the positively-regulated promoters was not present in the chromosome of non-virulent *E. coli* laboratory strains, DH5α and HB101. Further analysis of this positively-regulated promoter in EPEC showed that it resided within a 5.8-kb sequence that is not present in *E. coli* K12. This locus was found to contain four ORFs: ORF2 and ORF 3 are located upstream of ORF1 in the opposite orientation. Southern hybridization with a probe from this locus showed that some enterohemorrhagic *E. coli* also harbor this sequence, as well as some EPEC and RDEC strains. Both transcription of ORF1 and ORF2-ORF3 are greatly reduced in a *bfpT* mutant, suggesting that these newly discovered EPEC chromosomal genes are controlled by the *bfpTVW* operon and are likely to co-regulated with other virulence genes of EPEC. Their function is now under study using in vitro and in vivo assays and knockout mutants of the ORF1-ORF2 locus.

PMN ARE THE FIRST LINE OF DEFENSE AGAINST *SALMONELLA* WITH VIRULENCE PLASMIDS, WHILE MACROPHAGES ARE THEIR PLACE OF REFUGE.

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For many years investigators have focused on macrophages as the cells primarily responsible for resistance to *Salmonella*, although in vitro, macrophages have very little bactericidal activity against *Salmonella*. We used the monoclonal antibody RB6-8C5 to make B/c.D2 ($ity^{r/r}$) congenic mice neutropenic, and one day later infected them i.v. with *S. dublin*. We measured CFU in liver, spleen, and blood at intervals after infection. On day one, when there were not yet any PMN in the liver of normal mice, CFU were identical in control and neutropenic mice. From then on *S. dublin* multiplied in neutropenic mice about twice as rapidly as in control mice, and the former died. We allowed the mice to recover from neutropenia, and they recovered their ability to resist *S. dublin* infection. In contrast, the isogenic cured strain LD842 grew only about 50% more rapidly in neutropenic congenic mice for the first three days after infection; thereafter growth in the two groups was similar. Neutropenic B/c mice were killed by as few as 50 CFU of LD842, so their macrophages appeared to be unable to suppress the growth of plasmid-cured bacteria; control mice survived infection with $10^4$ LD842. Neutropenic B/c mice were not susceptible to infection with macrophage-sensitive mutants such as *purA-* and *phoP-* strains. In vitro, PMN from mice and humans killed 95% of an inoculum of *S. dublin* and *S. typhimurium* in only 30 minutes, in a complement dependent manner. These results lead us to conclude that complement-opsonized *Salmonella* are easily killed by PMN in vivo, and that *Salmonella* survive by entering macrophages, to which they have adapted. Numerous regulated genes are needed for successful macrophage adaptation, but the *spv* genes are needed for bacterial multiplication. The *nramp1* mutation allows Salmonella without *spv* genes to multiply, but not bacteria that have mutations in so-called macrophage survival genes. This may be because many of those genes were found by infecting B/c mice.

## Human Defense against bacterial enteric pathogens: inferences from successes and failures of live oral vaccines

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Human enteric pathogens are one of the leading causes of morbidity and mortality worldwide. In the past 10 years, impressive progress has been made towards unravelling the complex pathogenic mechanisms that allow bacterial enteropathogens to colonize their host. For some of them, such as *Salmonella* spp, *Vibrio cholerae*, *Shigella* spp., *E. coli*, and *Listeria* spp., the contribution of genes involved at different stages of the infectious process have been characterized and mutants affected in supposedly key functions were evaluated as candidate vaccines. Indeed, it is broadly accepted that only live attenuated vaccines administered orally may confer high protection levels against such pathogens..

The host immune responses induced by infection have also been partially deciphered. However, most available data result from animal models that can only approximate infections with human pathogens. Accordingly, the experimental results issued from such models often proved disappointing when applied to the development of human vaccines, as they led to candidate attenuated vaccine strains showing either weak immunogenicity and low protection level, or being too reactogenic. Consequently, most vaccine projects fail at the stage of the very first phase 1 study.

Our Institute has a long tradition in the development of vaccines against bacterial enteric diseases. In-house research recently led to several candidate carrier-based vaccines against *V. cholerae* and *Shigella* spp. In order to better understand the host response to enteric pathogens, we were interested to gain information from the successes and failures of established and candidate oral vaccines tested in humans. In this presentation, vaccine-induced immunity and the degree of conferred protection will be compared to disease-induced immunity. The interplay of genetics and physiology for the efficacy of vaccine strains and the implications for the design of effective vaccines against enteric diseases will be discussed.

INTERACTION OF FIMBRIATE *HAEMOPHILUS INFLUENZAE* TYPE B WITH HEPARIN-BINDING MATRIX PROTEINS

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*Haemophilus influenzae* type b (Hib) strains cause meningitis in young children. The bacteria adhere to and colonize oropharyngeal epithelial cells. Invasion into circulation is belived to take place in nasopharynx, whereas the choroid plexus is the portal entry of bacteria from blood to cerebrospinal fluid. Our recent studies have shown that Hib strain 770235 recognizes various receptors in subendothelial extracellular matrix (ECM). Hib also express plasminogen receptors (PlgRs) on their surfaces, and our *in-vitro* assays have shown that PlgRs promote degradation of and penetration through ECM by Hib. ECM receptors for Hib include two heparin-binding proteins, HB-GAM, a developmentally-regulated ECM component, and fibronectin (FN). Binding of Hib to these targets is inhibited by high concentration of heparin, and within the FN molecule, the binding is targeted to two heparin-binding fragments of FN. Analyses with purified fimbriae, mutant Hib strain as well as fimbriae-expressing recombinant *E. coli*, indicated that the binding is based on a protein-protein interaction. Hib PlgRs and FN-adhesiveness contribute to degradation of FN and subendothelial ECM, and our hypothesis is that these characteristics promote invasion of Hib to circulation and subsequently to cerebrospinal fluid.

COMPARED TO RAPID AND EXTENSIVE HYDROLYSIS OF BACTERIAL PHOSPHOLIPIDS, PROTEINS, AND RNA, $^{3}$H-ACETATE-LABELED LIPOPOLYSACCHARIDES OF *ESCHERICHIA COLI* INGESTED AND KILLED BY GRANULOCYTES UNDERGO SLOW AND LIMITED DEACYLATION. Yvette Weinrauch, Seth Katz, Peter Elsbach, Robert Munford*, and Jerrold Weiss

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The mammalian host appears to have a limited capacity to degrade isolated bacterial lipopolysaccharides (LPS). To explore the extent to which LPS of intact bacteria killed by host cells are subject to hydrolytic attack, we used $^{3}$H-acetate-labeled *E. coli* LCD25 (a strain that exclusively incorporates acetate into fatty acids of both phospholipids and the lipid A moiety of LPS). The radiolabeled bacteria were incubated with the cells (>90% granulocytes) and/or the extracellular fluid of a sterile inflammatory peritoneal exudate elicited in rabbits. Nearly complete loss of viability was accompanied by 80–90% hydrolysis of bacterial phospholipids and ~40% degradation of specifically radiolabeled protein and RNA that reached a plateau within 1 h. In contrast, release of LPS-associated fatty acids did not exceed 10%, did not become apparent until after 4 h., and progressed slowly for 20 h. During incubation with isolated LPS, combined intra- and extracellular hydrolysis was similarly slow and limited. Thus, both cellular and extracellular host-defense systems mobilized during inflammation effectively contribute to overall bacterial destruction, but are incapable of rapid and extensive disassembly of bacterial LPS. These results raise questions about the means by which the host detoxifies and disposes of these bacterial components.

# EFFECT OF *IN VIVO* MACROPHAGE DEPLETION ON *SALMONELLA ENTERICA VAR. TYPHIMURIUM* INFECTION IN MICE

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The pathogenic Salmonellae are regarded, with *Mycobacteria spp.*, *Brucella spp.* and *Listeria spp.*, as the classical facultative intracellular bacterial pathogens. The grouping resulted from early immunology studies which suggested a common and major role for T cells, later shown to be of the TH1 subtype, in the protective immune responses against the prototypic pathogens in each genus and led to investigations of the *in vitro* interaction between *S. Typhimurium* and macrophages, a major mediator of TH1 effector activity in mycobacterial infections. The studies revealed that mutants of *S. Typhimurium* which survived poorly inside macrophages *in vitro*, were attenuated *in vivo*, and the findings were used to support the theory that Salmonellae are 'intramacrophage' pathogens which replicate inside macrophages in naive animals. We used the liposome-based macrophage depletion technique to study the interaction between *S. Typhimurium* and macrophages *in vivo*. Depletion of macrophages prior to, and during, intravenous infection by an aromatic mutant of *S. Typhimurium* SL1344 (Δ*aroA*, Δ*aroD* ie. BRD509) did not alter distribution of the bacterium within the spleen or liver, or the bacterial clearance kinetic; gentamicin treatment of infected mice revealed that the bacteria were intracellular. The humoral responses to Salmonella lysate, and a surrogate carried antigen (C fragment of tetanus toxoid), were also unaffected by macrophage depletion however T cell responses to C fragment were, paradoxically, raised in macrophage-depleted mice. BRD509 immunised, depleted and non-depleted mice, were challenged with wild-type *S. Typhimurium* (SL1344) to determine the role of macrophages in the induction and effector function of the protective immune response. These studies revealed that macrophages have little, if any, role in the induction of humoral responses following intravenous immunisation with BRD509. Macrophages participated in BRD509-mediated protection, however another macrophage-independent mechanism must also be involved in controlling replication of SL1344 in BRD509-vaccinated animals.. Our data, together with recent studies showing that *S. Typhimurium* entry into macrophages leads to apoptosis, suggests that the persistent cellular home of the bacterium is unlikely to be macrophages. Further, our studies suggest that while macrophages participate in the protective response, other factor(s) are centrally involved in the protective effector response induced by immunisation with aromatic mutants of *S. Typhimurium*.

# THE N-TERMINUS OF *PASTEURELLA MULTOCIDA* TOXIN UNCOUPLES THE $G_q\alpha$-PROTEIN-MEDIATED $IP_3$ SIGNALING PATHWAY IN *XENOPUS* OOCYTES.

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*Pasteurella multocida* is associated with bovine respiratory disease and progressive atrophic rhinitis (PAR). *P. multocida* toxin (PMT) is an important virulence factor of *P. multocida*, and purified PMT alone is sufficient to experimentally induce PAR and symptoms of pneumonia in animals. We used voltage-clamped *Xenopus* oocytes to demonstrate direct PMT-mediated stimulation of $PIP_2$-PLC$\beta$1. Microinjection of native and recombinant PMT induced an inward, two-component $Cl^-$ current, similar to that evoked by microinjection of $IP_3$ through intracellular $Ca^{2+}$ mobilization and $Ca^{2+}$ influx through plasma membrane $Ca^{2+}$ channels. Antibodies directed against PMT, PLC isoforms, and various G-proteins, were used to demonstrate the specific involvement of the $G_q\alpha$-protein-coupled PLC$\beta$1-$IP_3$ pathway in PMT action. Overexpression of $G_q\alpha$ in oocytes enhanced the PMT response by >30-fold, whereas introduction of anti-sense $G_q\alpha$ cRNA reduced the response 7-fold, demonstrating that the intracellular target of PMT is the $\alpha$ subunit of the $G_q$-protein, which is transiently activated by PMT to stimulate the effector PLC-$\beta$1 activity. PMT acts preferentially on the monomeric $G_q\alpha$ subunit and not the heterotrimeric $G_q\alpha\beta\gamma$ complex. Prior treatment with pertussis toxin significantly enhanced the PMT response in a time-dependent manner. PMT apparently irreversibly uncouples the $G_q$-protein-PLC$\beta$1 signaling system because the transient activation is followed by irreversible inactivation. Antibodies directed against an N-terminal peptide of PMT inhibited the toxin-induced currents, implicating the N-terminus of PMT is important for toxin activity. A hexahistidine fusion peptide placed at the N-terminus blocked activity of the toxin, but removal by proteolysis fully restored activity. Using our *Xenopus* oocyte system to screen for activity, deletion analysis revealed that the N-terminus of PMT contains the activity domain.

IDENTIFICATION OF BACTERIAL $CD8^+$ T CELL ANTIGENS USING AN EXPRESSION CLONING SYSTEM

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We hypothesize that effective $CD8^+$ T cell mediated immunity against an intracellular bacterial pathogen results from recognition of peptide epitopes derived from proteins secreted from the bacteria. Experiments designed to test this hypothesis involve the development of an expression cloning system to identify *Listeria monocytogenes* (LM) proteins that contain epitopes recognized by $CD8^+$ T cells. Once novel epitopes are identified, the bacterial localization of the protein will be determined by the ability of the protein to co-localize with proteins of known compartmentalization. $CD8^+$ T cells specific only for the identified antigens will be generated and used to determine the relationship between antigen compartmentalization and protective immunity. This poster details the development of the expression cloning system.

The shuttle vector pcDNA3 was modified to allow the expression of bacterial gene fragments in eukaryotic cells. To determine whether COS7 cells could appropriately express bacterial gene fragments, *E. coli* β-galactosidase was used as a model antigen system. The *E. coli* β-gal protein contains a $CD8^+$ T cell epitope (a.a. 876-884) presented by the murine H-$2L^d$ MHC class I molecule. COS7 cells cotransfected with a plasmid encoding H-$2L^d$ and the modified pcDNA3 encoding a.a. 760-991 of β-gal (pβgal 760-991) were able to stimulate specific production of TNF by β-gal specific murine $CD8^+$ T cells. This indicates that COS7 cells are capable of expressing, processing, and properly presenting a bacterial peptide in the context of a murine MHC class I molecule in a manner that promotes antigen specific TNF production by a murine $CD8^+$ T cell line.

A library containing small random fragments of LM DNA was constructed in the modified vector. This library will be screened with polyclonal $CD8^+$ T cells which recognize unknown LM antigens. In order to efficiently screen the library for novel $CD8^+$ T cell antigens it will be necessary to make pools of plasmid DNA. To determine the maximal pool size that still allows for the detection of antigen-encoding plasmids, the model antigen encoded by pβga760-991 was diluted into pools of plasmids from the LM library. DNA from these pools was used to transfect COS7 cells. TNF production by the βgal specific $CD8^+$ T cells was detectable even when pβgal760-991 was diluted 75-fold with the random plasmids, suggesting that pools of 75 plasmids can be used for the initial screen of the LM library. Furthermore, experiments with mixed $CD8^+$ T cell populations revealed that only 3% of the $CD8^+$ T cells needed to be specific for the diluted β-gal epitope indicating that polyclonal $CD8^+$ T cell lines with multiple antigen specificities will be useful for screening the LM DNA library.

# ROLE OF RPOS IN STRESS SURVIVAL AND VIRULENCE OF VIBRIO CHOLERAE

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*Vibrio cholerae* is known to persist in aquatic environments under nutrient limiting conditions. To analyze the possible involvement of the alternative sigma factor *rpoS*, which is shown to be important for survival during nutrient deprivation in several other bacterial species, a *V. cholerae rpoS* homolog was cloned by functional complementation of an *Esherichia coli* mutant using a wild type genomic library. Sequence analysis of the complementing clone revealed an 1007 bp open reading frame which is predicted to encode a 336 amino acid protein that shares 71 to 63 % overall identity to sequences reported for *rpoS* gene products. To determine the role of *rpoS* in *V. cholerae*, we inactivated *rpoS* by homologous recombination. *V. cholerae* strains lacking *rpoS* are impaired in their ability to survive diverse environmental stresses, including hydrogen peroxide, high osmolarity and carbon starvation. These results suggest that *rpoS* may be required for the persistence of *V. cholerae* in aquatic habitats. In addition, *rpoS* mutants are altered in their ability to secrete cholera toxin and proteases. However, *rpoS* is not critical for in vivo survival as determined by infant mouse competition assay.

## CONSTRUCTION AND CHARACTERISATION OF *SHIGELLA FLEXNERI dsb* MUTANTS: AN INVESTIGATION INTO THE ROLE OF THIOL:DISULPHIDE OXIDOREDUCTASES IN *SHIGELLA* PATHOGENICITY

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The periplasmic protein DsbA of *Shigella flexneri* has been shown to catalyse formation of a single intramolecular disulphide bond in the outer membrane protein, Spa32. Failure to form this disulphide bond leads to the failure of Spa32 to facilitate release of Ipa invasins, reducing invasiveness. We have postulated that the multiple periplasmic disulphide bond catalysts encoded by *dsb* genes all play crucial roles catalysing modification of other cellular factors necessary for invasion, and facilitating bacterial multiplication in the reducing cytosol of eucaryotic cells after *Shigella* escapes from phagocytic vacuoles. To test this hypothesis, we have constructed *dsbA, C* and *D* mutants of *S. flexneri* 5 M90T by P1 transduction and allele exchange, and analysed their virulence properties *in vitro*. All the *dsb* mutants were less invasive than wild type in HeLa cells and J774 mouse macrophages. In each case viable counts of bacteria released from HeLa cells 2 hours post infection fell, from 30 to 60 fold in the case of different *dsb* mutants compared to wildtype. Giemsa staining of J774 cells one hour post infection showed that internalised *dsb* mutants had dropped 10-20 fold. This preliminary data argues a role for each of the *dsb* genes in *Shigella* pathogenicity. Investigations are underway to assess fully the virulence of the mutants using other *in vitro* and *in vivo* assays, and to understand the underlying mechanisms of attenuation.

GENES WHICH INHIBIT GENETIC RECOMBINATION BETWEEN *SALMONELLA TYPHIMURIUM* AND *SALMONELLA TYPHI* ALSO AFFECT VIRULENCE IN VIVO. Thomas Zahrt, Jennifer Neitzer, and Stanley Maloy. Department of Microbiology, University of Illinois, Urbana, IL 61801.

The *Salmonellae* are responsible for a variety of diseases ranging in severity from an often deadly systemic infection, to a simple, rate-limiting gastroenteritis. Two of the most widely studied species which show this variation in disease symptoms in humans are *S. typhi* and *S. typhimurium*. *S. typhi* is a human-specific pathogen responsible for typhoid fever. In contrast, *S. typhimurium* infects a variety of hosts and causes gastroenteritis in humans. However, *S. typhimurium* can cause a disease which is analogous to human typhoid fever in mice. We are using *S. typhi* and *S. typhimurium* as a model for identifying the determinants which contribute to host specificity differences between closely-related bacterial species.

The introduction of "murine" host specificity genes from *S. typhimurium* into *S. typhi* requires the inactivation of the *mutS* and *recD* gene products. However, mutants of *S. typhimurium* carrying mutations in both *mutS* and *recD* appear to be altered for virulence *in vivo*. For example, competition of a wild-type strain and *mutS recD* mutant initially inoculated intraperitoneally in a 1:1 ratio, results in a $10^3$-$10^4$-fold under-representation of the *mutS recD* mutant in the spleen or liver just prior to death. However, *mutS recD* mutants are not attenuated, because the $LD_{50}$ of the mutant remains similar to that of the wild-type strain. In addition, the time required for the mutant to elicit disease symptoms in mice is twice as long as it takes the wild-type strain. The inactivation of either *mutS* or *recD* does not result in any detectable virulence phenotype *in vivo*.

Our results suggest that mutations in *mutS* and *recD* negatively affect the ability of strains grown in nutrient-abundant conditions to adapt to growth in nutrient-limiting conditions. The effect of these mutations on virulence is probably an indirect effect of the nutrient-limiting conditions experienced *in vivo*, rather than the disruption of genes normally involved in bacterial pathogenesis.

p15s, RABBIT CATHELICIDIN HOMOLOGUES, EFFECTIVELY KILL SERUM-RESISTANT *ESCHERICHIA COLI* IN SYNERGY WITH COMPLEMENT AND OTHER COMPONENTS OF INFLAMMATORY FLUID.

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The cell-free ascitic fluid (AF) of sterile inflammatory peritoneal exudates induced in rabbits contains several antimicrobial components active against serum-resistant *E. coli* K1/R. Neutralizing antiserum against the Bactericidal/ Permeability-Increasing protein (BPI) eliminates this activity. However, BPI levels alone in AF are insufficient. Full antimicrobial activity depends on synergy with p15s (secreted from the secondary granules of the granulocyte) as judged from *in vitro* reconstitution with purified proteins in simple media. Here we have further examined the antibacterial activity of purified p15s in AF depleted of endogenous cationic antibacterial proteins, including BPI, p15s and phospholipase A2 ($PLA_2$), by cation-exchange chromatography. The unbound fluid (AFUB) contains $^{3}$99.5% of total AF protein, including complement and serum albumin. Killing of *E. coli* K1/R *lux*$^{+}$ was measured by luminometry. Neither AFUB nor p15s alone affected bacterial viability, but p15s added to 90% AFUB, at levels $\leq$ those found in unmodified AF (~500 nM), in a dose and time dependent fashion killed in the absence of BPI. Killing was accelerated when purified AF $PLA_2$ was also added at levels found in AF. In AFUB from complement (C6)-deficient rabbits the p15s ($\pm$ $PLA_2$) are inactive against serum-resistant bacteria. The full restoration of activity by repletion of C6 reveals that the p15s act in synergy with the complement system, providing yet another example of the integration of humoral and cell-mediated innate defenses.

# MODULATION OF *LISTERIA MONOCYTOGENES* PC-PLC ACTIVITY THROUGH SINGLE AMINOACID SUBSTITUTIONS AND COMPLEMENTATION BY THE *BACILLUS CEREUS* HOMOLOG

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*Listeria monocytogenes*, a facultatively intracellular gram positive pathogen, secretes two phospholipases C, PI-PLC and PC-PLC, which play overlapping accessory roles in the bacterium's ability to escape from phagosomes, grow intracellularly and spread from cell to cell. Encoded by *plcB*, PC-PLC prefers phosphatidylcholine (PC), but also cleaves other phospholipids, such as sphingomyelin (SM). PC-PLC contains $Zn^{2+}$ in its active site and is activated upon secretion by either a listerial metalloprotease (Mpl) or other proteases, presumably of host origin.

Zinc phospholipases partly homologous to PC-PLC have been characterized in *Clostridium perfringens* (α-toxin) and *Bacillus cereus* ($PLC_{Bc}$). The α-toxin is hemolytic, active on both PC and SM and has been characterized by a series of point mutations. $PLC_{Bc}$ has been crystallized and compared to PC-PLC, exhibits an approximately 5-fold higher activity on PC, but very low sphingomyelinase activity. Based on these studies, we constructed three sets of *Listeria* PC-PLC mutants: (a) Single aminoacid substitutions H69G and H118G targeted two of the conserved $Zn^{2+}$ coordinating histidines. Identical substitutions in the α-toxin lead to complete or 99% inactivation, respectively. (b) Substitutions D4E and H56Y, based on structure data and sequence alignments, should convert the PC-PLC active site to a $PLC_{Bc}$ active site. (c) *Listeria* expressing $PLC_{Bc}$ instead of PC-PLC. The mutants were then analyzed for changes in enzymatic activity on PC and SM in vitro and for biological function in cell culture models of infection.

Both the H69G and H118G substitutions in PC-PLC lead to stable, but inactive proteins in vitro. Inside host cells, their phenotypes resembled a *plcB* in-frame deletion, however the mutant proteins seemed to be prone to degradation. The D4E, H56Y and $PLC_{Bc}$ mutant proteins were stably expressed both in vitro and intracellularly. D4E increased both activity on and specificity for PC, while H56Y showed higher activity on both PC and SM, with reduced specificity for PC. The biochemical characteristics of $PLC_{Bc}$ expressed by *Listeria* were in agreement with published $PLC_{Bc}$ data. During early intracellular growth, D4E and $PLC_{Bc}$ mutants performed significantly better than the wild type enzyme, while H56Y showed a significant defect. In assays for cell-to-cell spread, H56Y and D4E had close to wild type characteristics, while the spreading efficiency of $PLC_{Bc}$ was significantly lower. These studies emphasize the species-specific features of listerial PC-PLC, which are important for growth in a mammalian cell.

# REGULATION OF SALMONELLA TYPHIMURIUM INVASION GENES: TO INVADE OR NOT TO INVADE, THAT IS THE QUESTION.

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Salmonella invade intestinal epithelial cells during infection. Models of Salmonella disease suggest that bacterial entry into these non-phagocytic cells is important for infection of the intestinal mucosa as well as for bacterial penetration of the intestinal epithelial barrier. Interestingly, in vivo studies also suggest that Salmonella invasion does not occur at systemic sites and does not contribute to systemic stages of infection.

We are investigating how Salmonella invasion is restricted during infection. Our results suggest that the regulation of Salmonella invasion genes is an important mechanism which limits bacterial invasion in vivo. Salmonella typhimurium invasion genes are located within a 40 Kb pathogenicity island. Most of the invasion genes encode a type III secretion system while other genes encode the secreted proteins that are thought to directly interact with the epithelial cells and trigger the invasion process. In vitro studies show that the expression of S. typhimurium invasion genes is regulated by many environmental and regulatory factors. Interestingly, S. typhimurium will not express invasion genes unless a specfic set of regulatory conditions have been met. We are investigating the importance of invasion gene regulation in Salmonella pathogenesis.

Bacterial factors that mediate the complex regulation of S. typhimurium invasion genes have been identified. Transcriptional activators that are encoded on the 40 Kb pathogenicity island, HilA and InvF, comprise a regulatory cascade that controls the expression of invasion genes. HilA appears to directly activate the transcription of invF and the genes that encode the type III secretion system. InvF appears to specifically activate the transcription of the genes that encode the secreted invasion proteins. Global regulatory factors are also involved in modulating the expression of invasion genes. Ultimately, these studies will help us understand how Salmonella decide to infect non-phagocytic host cells during infection.

# A LOCUS OF *SALMONELLA TYPHIMURIUM* INVOLVED IN PEYER'S PATCH SURVIVAL

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*Salmonella typhimurium* causes a typhoid-like condition in BALB/c mice, which can be used as a model for the human infection caused by *Salmonella typhi*. The IVET technique (*in vivo* expression technology) was designed to identify operons that are transcriptionally active only when the bacterium is in the host. We have used this system to identify novel virulence genes of *Salmonella typhimurium* required at various stages of the infection process. In particular, we have identified a locus of *Salmonella typhimurium* that is involved in survival or growth in Peyer's patches, an early step in the infection process. Fusions to this locus are transcriptionally active only during the first stages of infection in the small intestine, and are not expressed during systemic infection. A strain containing a null mutation in this locus is defective in survival or growth in a Peyer's patch and has a 5-fold increase in oral $LD_{50}$. This mutation has no effect on the $LD_{50}$ following intraperitoneal inoculation. Sequence analysis indicates that this locus consists of a single open reading frame of approximately 1200 bp located on a cryptic lambdoid phage. It encodes a transposase-like protein that has homologs in a variety of organisms, ranging from Anabaena to Sulfolobus. In two organisms, *Dichelobacter nodosus* and *Helicobacter pylori*, homologs of this gene are associated with virulence determinants. Future studies will include determining the regulation of the operon both *in vitro* and *in vivo*, and characterizing the mode of action of the gene product within the Peyer's patch.

**T CELL RESPONSES TO *SALMONELLA*: ANTIGENIC SPECIFICITY AND ACTIVATION OF MACROPHAGE FUNCTION.** Brad T. Cookson and Michael J. Bevan, Departments of Laboratory Medicine, Microbiology, Immunology and HHMI, University of Washington, Seattle, WA

Oral immunization of susceptible mice with viable attenuated *Salmonella typhimurium* (ST) provides long lasting protection against lethal enteric fever caused by virulent ST. CD4+ T cells, which contribute to this immunity, recognize processed antigens presented as peptides bound to class II MHC molecules on the surface of antigen presenting cells (APC). Using a genetic approach, we have identified the bacterial flagellar filament protein (FliC) as the dominant recall antigen recognized by T cells from protectively immunized C3H/HeJ mice. Generation of T cells with this specificity requires immunization and they can be recovered for at least 7 months afterwards. Sequential truncation of MalE-FliC fusion proteins and synthetic peptides indicate FliC residues 339-350 bound by class II MHC are the minimal antigenic structure capable of stimulating 7.4.8, a CD4+ T cell clone derived from ST-infected mice. FliC 339-350 is presented by infected macrophages, and presentation, but not bacterial uptake, is greatly enhanced by pretreatment of macrophages with IFN-g. Correspondingly, chemically-fixed macrophages pretreated with IFN-g present pre-processed peptide antigen much more efficiently than fixed macrophages without IFN-g pretreatment, largely due to higher surface expression of MHC by the IFN-g treated macrophages. Interestingly, equivalent T cell activation occurs when peptide is presented by viable macrophages either untreated or pretreated with IFN-g, despite large differences in class II MHC expression by the two macrophage populations. These data suggest antigen-stimulated T cells interact with viable macrophages to enhance APC functions. T cell clone 7.4.8 produces both IL-2 and IFN-g, cytokines known to activate macrophages, yet neither of these cytokines nor supernatants from activated T cells can account for the augmented macrophage APC function observed in this system. We have determined that cell-cell interaction between activated T cells and viable macrophages enhances macrophage APC function, and the potential mechanism for this T cell-mediated macrophage activation will be discussed. These data point to the importance of both cytokine and cell-to-cell signaling in stimulation of T cells responding to intracellular pathogens like *Salmonella.*

INTEGRIN CLUSTERING AND INVASIN-PROMOTED BACTERIAL INTERNALIZATION INTO MAMMALIAN CELLS.

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Enteropathogenic Yersinia are efficiently internalized by cultured mammalian cells as a result of binding of the bacterial invasin protein to mammalian integrin receptors. Invasin is also required for efficient translocation of the microorganism from the lumen of the intestine into the Peyer's patch, and bacteria are found in the rare M cells in the epithelium that express the appropriate integrin receptors.

Our laboratory has investigated the determinants that allow invasin-mediated bacterial internalization into mammalian cells. Uptake appears to occur via a zippering mechanism. Internalization requires high affinity binding to the integrin receptor via the carboxyl terminal of invasin. Efficient internalization also requires a region of invasin located N-terminal to the cell binding domain that enhances bacterial uptake, presumably by stimulating local clustering of receptor. Particles coated at low concentration with a purified protein derivative containing these two domains are efficiently internalized, whereas derivatives harboring only the cell adhesion domain require large amounts of protein to promote the same level of internalization.

Analyses of integrin determinants necessary for internalization indicate that the short cytoplasmic tail of the receptor is necessary for internalization. Mutations in the tail that cause defects in internalization appear to prevent the receptor from entering the endocytic pathway. In addition, clustering of receptor by invasin-bound appears to result in a signal cascade that is required for bacterial internalization. At least one GTP-binding protein is required for this event, as are the kinases FAK and a Src family member. Alterations of the activity of these factors by dominant negative mutation or toxin modification results in depressed bacterial uptake. A model that integrates the roles of endocytosis factors, signaling molecules and cytoskeletal proteins in bacterial internalization will be presented.

## IDENTIFICATION OF NOVEL CHROMOSOMAL LOCI AFFECTING *YERSINIA ENTEROCOLITICA* PATHOGENESIS BY GENETIC SELECTION IN AN ANIMAL HOST

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Pathogenic species of the genus *Yersinia* have a marked tropism for lymphoid tissue during the early stages of infection. Bacterial survival at this site determines whether the disease is localized or systemically progresses leading to a high rate of mortality. Several plasmid encoded virulence genes are known to be required for survival and pathogenesis, but the contribution of chromosomal genes has been largely unexplored. This study represents the first intensive effort to characterize and determine the function of *Yersinia* chromosomal genes expressed in lymphoid tissue after intragastric infection. Strains harboring *cat* fusions expressed in the host were isolated from Peyer's patch tissue of mice intragastrically infected and treated with chloramphenicol (Cm); genes identified in this manner were designated *hre* for host responsive element. The *hre*::*cat* stains that were Cm resistant *in vivo* (in mouse tissue) and Cm sensitive *in vitro* (on laboratory media at 26°C) were identified and shown to consist of 61 different allelic groups. The *hre*::*cat* fusions from 48 of the allelic groups were cloned and characterized by DNA sequence analysis. The results identified genes necessary for iron acquisition, protection from environmental stresses, biosynthesis of cell envelope components and other diverse metabolic activities. However, the DNA sequence of many clones had no homology to other known genes. Insertion mutations were constructed for four *hre* genes and the resulting *Y. enterocolitica* mutants were tested in the mouse model for effects on pathogenesis. All of the mutant strains were affected for virulence when assayed for survival in host tissues and $LD_{50}$ analysis. A more detailed characterization of one of these *hre* indicates it encodes a protease homologous to the S8 familily of subtilisin-like serine proteases. We are currently investigating the role of this protease in *Y. enterocolitica* pathogenesis.

# A FUNTIONAL TYPE III PROTEIN TRANSLOCATION PATHWAY IN *YERSINIA PSEUDOTUBERCULOSIS* IS REQUIRED FOR INHIBITION OF MACROPHAGE TNFα EXPRESSION AND DOWNREGULATION OF P38 MAP KINASE

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Exposure of macrophages to lipopolysaccarhide (LPS) leads to activation of several mitogen-activated protein (MAP) kinases, including JNK and p38, and the expression of the proinflammatory cytokine TNFα. Previous studies have suggested that one or more Yops that are secreted by a type III pathway in pathogenic *Yersinia* function to suppress LPS-mediated expression of TNFα by macrophages. In support of this idea, we found that TNFα expression was suppressed when J774A.1 murine macrophages were infected with a wild-type *Y. pseudotuberculosis* strain, but not with an isogenic *ysc* mutant defective for secretion of all Yops. Infection of macrophages with the *ysc* mutant lead to weak activation of JNK and strong, sustained activation of p38. Conversely, infection with wild-type bacteria caused strong, transient activation of JNK and downregulation of p38. Therefore, suppression of TNFα was most closely linked to downregulation of p38. Analysis of strains defective in one or more Yops indicated that YopE, YopH, YopK, YopM and YpkA were not required, while YopB was required for inhibition of TNFα expression and downregulation of p38. Our results suggest that one or more unidentified Yops, that are dependent upon YopB for translocation into host cells, function to suppress macrophage expression of TNFα through a process that involves downregulation of p38 MAP kinase.

**Neutrophils exhibit complex pattern of gene expression during interaction with pathogenic bacteria.** Y.V.B.K. Subrahmanyam*, Yatindra Prashar#, Nancy Hoe+,Constance Whitney@, Jon D.Goguen+, Peter E.Newburger@ and Sherman M.Weissman*.

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We have used the display of 3'-end restriction fragments of cDNAs (Proc. Natl. Acad. Sci.USA., 1996 Vol.93, 659-663) to study the pattern of changes in gene expression in human peripheral blood neutrophils during interaction with selected species of pathogenic and non-pathogenic bacteria: *Yersinia pestis* (the causative agent of plague, enterohemorrhagic *E. coli* (EHEC), and non-pathogenic *E. coli* K12. Although neutrophils are terminally differentiated cells that have relatively low levels of mRNA and are generally considered to respond to bacterial challenge primarily by activation or release of pre-formed proteins, a large number of cDNA bands were observed for each of the bacteria tested. Moreover, the pattern of these bands was strongly dependent on the identity of the bacteria involved. Nearly 100 cDNAs were recovered from the display gels, cloned, and sequenced. Twenty-nine of these were homologous to sequences of unknown significance contained only in the EST database, and twelve sequences--for which no homologies were found--appear to novel. More interestingly, 27 sequences were homologous to genes with known protein products. These products include, among others, a vacuolar ATPase subunit, the ras-mediated protein rab, bcl-2, bcl-3, IL-8, and LD78 (the human homologue of mouse MIP 1α). In some cases, the bacteria-specific differences observed can be logically related to pathogenesis. For example, virulent Y. pestis did not induce either IL-8 or MIP1α--both important inflammatory cell chemoattractants--even though these genes were strongly induced by both of the *E. coli* strains tested and by some avirulent *Y. pestis* mutants. The observed suppression of IL-8 induction is consistent with previous observations made by assay of the IL-8 peptide and by Northern blotting for IL-8 mRNA, confirming the utility of differential display in analysis of bacteria-cell interactions. Additional confirmation was provided by quantitative RT-PCR of neutrophil mRNA, using primers homologous to the coding sequences of selected genes. In all cases tested, results were in good agreement with the intensity differences observed via differential display. We are currently applying this technology to establish the role of specific *Y. pestis* genes in the control of neutrophil gene expression.

# REGULATION OF *SALMONELLA TYPHIMURIUM* INVASION GENE EXPRESSION BY *barA*

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Expression of *Salmonella typhimurium* invasion genes is regulated through a complex interaction of environmental and genetic factors. To date, *hilA*, *sirA*, and *invF* have been identified as positive regulators, and *phoPQ* as a negative regulator, of invasion gene expression. Here we report the existence of an additional layer of genetic regulation. We have studied a 19 kb chromosomal deletion located near *relA* at centisome 65, outside the SPI1 pathogenicity island. For bacterial cells growing in vitro under strongly inducing conditions, this deletion reduces invasion gene expression approximately 30- to 50-fold. Secretion of virulence proteins and invasion of cultured HEp-2 cells are also sharply reduced by the deletion. Further analysis suggests that the deletion affects at least two distinct positive regulators of invasion gene expression. One of the affected loci is the Salmonella homolog of *barA* (also called *airS*), an unusual member of the two-component regulatory family of *E. coli*. *E. coli barA* contains two transmitter domains and one receiver domain in a single protein. It has been proposed (Ishige et al., *EMBO J.* **13**:5195-202 [1994]) that the presence of two transmitter domains allows proteins with this kind of organization to integrate multiple environmental cues. BarA has also been recognized as a virulence factor in uropathogenic *E. coli* (Zhang and Normark, *Science* **273**:1234-1236 [1996]), and so may be a general regulator of virulence gene expression in enteric bacteria.

# HUMAN MACROPHAGES DEFINE ESSENTIAL ROLES FOR THE *SALMONELLA rpoS* AND *spvR* GENES IN PROMOTING INTRACELLULAR GROWTH

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We have developed a new assay to study the interaction of *Salmonella* strains with human monocyte-derived macrophages. In this system, peripheral blood monocytes are purified by density gradient centrifugation and differential adherence, then allowed to differentiate for five days in tissue culture wells supplemented with 15% autologous serum. Macrophages are infected with complement-opsonized *Salmonella* strains at a bacteria: cell ratio of 1:1, and residual extracellular bacteria are killed with gentamicin. Wild-type strains of *S. typhimurium* and *S. dublin* are able to proliferate in a proportion of the macrophages, resulting in detachment of the cells from the plastic surface. Cells containing viable bacteria are then recovered at various times by centrifugation of the culture fluid. The majority of the detached macrophages remain viable for up to 24 hours, as determined by both trypan blue exclusion and fluorescent live/dead cell staining with commercial reagents. The majority of the bacteria are also viable up to 24 hours as determined by fluorescent staining and are protected from gentamicin by their intracellular location. Cytologic staining shows that many of the detached macrophages contain large numbers of bacteria, some with more than 50 *Salmonella* in a single cell.

The intracellular growth of *Salmonella* in this system is strictly dependent on the *rpoS* and *spvR* regulatory genes. Previous work has shown that both loci are required for *Salmonella* virulence in mice, but studies *in vitro* with murine macrophages have not revealed a difference between mutant and wild-type strains. *rpoS* encodes the alternative sigma factor, $\sigma^s$, which controls expression of the *spv* genes located on the virulence plasmid. *spvR* is a transcriptional activator that is essential for expression of the *spvABCD* operon. Mutations in *rpoS* or *spvR* abolish the ability of *S. typhimurium* and *S. dublin* to grow inside non-activated human monocyte-derived macrophages, but bacterial survival does not appear to be affected. These results directly demonstrate that proliferation of *Salmonella* strains within human macrophages is controlled by the *rpoS* and *spvR* regulatory mechanisms. This system will facilitate the determination of activating conditions and cytokines that alter the ability of human macrophages to control *Salmonella* growth.

TITLE: "**Accelerated Evolution: Emergence of Multidrug-Resistant Staphylococci and Pneumococci**"

**A. Tomasz, Rockefeller University, New York, New York**

SYNOPSIS: The introduction of massive quantities of antibacterial agents into the environment has created a novel evolutionary scale force selecting for the assembly of new genotypes, many of which involve acquisition of genetic determinants from heterologous sources or determinants affecting virulence-related properties. I shall discuss in my presentation three aspects of this phenomenon: (i) the origin; (ii) the mode of acquisition of resistance and/or virulence-related determinants; and (iii) the biological price of resistance (i.e., the requirements for the effective functioning of the acquired gene in the new host). I shall illustrate item (i) by recent studies that have allowed the identification of a close evolutionary relative (homologue) of the methicillin resistance determinant *mecA* in an animal species of staphylococcus; and by description of a new mechanism of vancomycin resistance in staphylococci. Item (ii) will be illustrated by the distribution and functioning of the genetic determinants *comA* and *comC* (controlling competence for genetic transformation) in natural populations of pneumococci and by the acquisition of new capsular determinants and the accompanying massive change in virulence properties. Item (iii): The abnormal chemistry of the cell wall often seen in penicillin-resistant pneumococci and the numerous chromosomal genes which appear essential for the optimal expression of β-lactam resistance in staphylococci (for instance, the need for an intact sigma-B operon) will be discussed in the context of the biological price of antibiotic resistance.

# THE CLUMPING FACTOR OF STAPHYLOCOCCUS AUREUS: A FIBRINOGEN-BINDING PROTEIN WITH INTEGRIN-LIKE CATION BINDING MOTIFS

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The clumping factor (ClfA) of *S.aureus* is a surface-associated protein which is covalently linked to the cell wall *via* its LPDTG sequence. ClfA promotes bacterial binding (i) to soluble fibrinogen to form cell clumps, (ii) to immobilized fibrinogen *in vitro*, (iii) to implanted medical devices which become rapidly coated with host plasma proteins, (iv) to blood clots and (v) to damaged heart tissue in a rat model for endocarditis. The ligand-binding domain is located between residues 220-559 in the N-terminal Region A. ClfA recognizes the same site in the γ-chain of fibrinogen as the platelet integrin αIIb/β3. The ligand-binding site on αIIb/β3 involves an EF-hand cation binding site. ClfA has several potential EF-hand motifs. Thus we investigated the role of divalent cations in ClfA-fibrinogen interaction. (i) $Ca^{2+}$ inhibits ClfA binding to the fibrinogen γ chain at concentrations above 3mM. (ii) Cation coordinating residues in EF-hand I were changed to alanine which reduced or eliminated fibrinogen binding. We propose that $Ca^{2+}$ competes with the fibrinogen γ chain at concentrations above 3mM and can both prevent the fibrinogen-binding and also displace bound ligand.

ClfA also possesses a cation binding MIDAS-like motif which occurs in the I-domain of leukocyte integrin αM/β2. We are currently investigating the role of the MIDAS in ligand binding.

**IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS* VIRULENCE GENES BY SIGNATURE-TAGGED MUTAGENESIS.**

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*Staphylococcus aureus* is a major human pathogen that causes a wide variety of diseases. However, relatively little is known of the molecular mechanisms of pathogenesis. Signature-tagged mutagenesis (STM) is a technique that permits the fate of a large number of bacterial mutants to be followed simultaneously in a single animal, thereby aiding the identification of virulence genes. We have applied STM to *S. aureus* and have screened mutants for attenuated virulence in a murine model of bacteraemia. An *S. aureus/E. coli* shuttle vector (pID408) was constructed that carries transposon Tn917 conferring resistance to erythromycin, a chloramphenicol resistance gene and a *S. aureus* temperature sensitive replicon. Plasmid pID408 was modified by creating 96 plasmids in which each transposon carries a different DNA tag that can be amplified and labelled efficiently, but which does not cross-hybridize with other selected tags. Virulent *S. aureus* strain RN6390 was transformed with each of these plasmids and 96 differently tagged plasmid transformants were stored at low temperature in the wells of a microtitre dish. For each round of mutagenesis, a replica microtitre dish is made and bacteria are grown at a non-permissive temperature in the presence of erythromycin to select against the plasmids and for the strains carrying chromosomally integrated transposons. Single colonies from each well that are erythromycin resistant and chloramphenicol sensitive are transferred to the corresponding wells of a third microtitre dish. Analysis of a large number of these RN6390 mutants showed that the single integrations of Tn917 occur at different genomic sites. Virulence tests were done in a murine model of bacteraemia using pools of 96 different mutants as inoculum. Virulent *S. aureus* mutants were isolated from the diseased animal and pooled to form the recovered pool. Tags in the recovered pool and inoculum pool were separately amplified, labelled and used to probe dot blots of the 96 different tags. Mutants with attenuated virulence were identified by reduced intensity or lack of hybridizing signals to tags from the recovered pool. An infinite number of mutants can be screened in this way with the same 96 tags. Analysis of about 1500 mutants has led to the provisional identification of 70 mutants with attenuated virulence. DNA regions flanking the transposons of these mutants were cloned and sequenced. Analysis of these sequences indicates that a variety of cell surface components are important for *S. aureus* growth in blood.

## THE INTERNALIZATION OF *STAPHYLOCOCCUS AUREUS* BY BOVINE MAMMARY EPITHELIAL CELLS LEADS TO THE INDUCTION OF APOPTOSIS

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*Staphylococcus aureus* is a pathogen with a broad host range, causing infections in humans and animals. We used an established bovine mammary epithelial cell line (MAC-T) to study the role of intracellular survival in the chronic persistence of staphylococci, leading to such diseases as toxic shock syndrome and bovine mastitis. *S. aureus* cells exhibited dose-dependent invasion of epithelial cells and intracellular survival. Electron microscopic examination of the infected cells indicated that the bacteria induced internalization via a mechanism involving membrane pedestal formation, and then appeared to escape into the cytoplasm following lysis of the phagosomal membrane. Internalization of *S. aureus* by the MAC-T cells was followed 2 to 4 hr later by the detachment and rounding of the epithelial cells, the appearance of a mottled cell membrane, and the vacuolization of the cytoplasm, all characteristics of cells undergoing programmed cell death (apoptosis). Also consistent with the induction of apoptosis was the generation of MAC-T cell DNA fragments that increased in size in increments of approximately 180 bps, and that the infected cells stained positive using the TUNEL assay. Thus, the data generated by this study demonstrates for the first time that internalized *S. aureus* cells escape the phagosome and induce apoptosis in nonprofessional phagocytes. These phenomena, along with widespread resistance to multiple antimicrobial agents, could be responsible for the persistence of certain types of staphylococcal infections.

## MICROBIAL SUPERANTIGENS AND THEIR MUTANTS - EFFECTS ON HUMAN EFFECTOR CELLS.

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Superantigens from different bacterial origin e.g. SEA, SEB, TSST-1 (*Staphylococcus aureus*) as well as ETA (*Streptococcus pyogenes*) are involved in the pathogenesis of several disease processes like atopic dermatitis, toxic shock, sepsis and Kawasaki syndrome. We analyzed the effects of different superantigens (SEA, SEB, TSST-1 and ETA) in contrast to their mutants (exchange of one or more amino acid residues in the T-cell receptor or MHC class II binding region), towards human peripheral blood mononuclesr cells (PBMC), with regard to immune parameters e.g. proliferation, cytokine expression/release (TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-13), Ig(A, E, G, M)-synthesis and CD23($Fc_{\varepsilon}RII$) expression. Our data clearly indicate, that both, the T-cell receptor- and the MHC class II binding region are required for efficient superantigen action. The exchange of one amino acid residue in the TSST-1 protein (H135A) results in the total loss of all immune activating features. With regard to B-cell activation we analyzed the membrane bound B-cell marker CD19 which is involved in the antigen presentation by MHC class II molecules. SEB induced CD19 mRNA expression in a dose dependent fashion. The depletion of CD 8 and CD14 positive cells from total PBMC did not affect CD19 mRNA expression by SEB, whereas depletion of CD4 positive cells abolished the capacity of SEB to induce CD19 mRNA expression. For CD19 protein expression measured by FACS analysis, identical results were obtained. The SEB T-cell receptor mutant activity was significantly reduced, whereas the SEB MHC class II binding mutant had no effects on CD19 expression. Thus, the mutant toxins may represent potential therapeutical agents for a variety of dsisease processes.

*ENTEROCOCCUS FAECALIS* CYTOLYSIN: A STRUCTURALLY NOVEL TOXIN THAT CONTRIBUTES TO THE PATHOGENESIS OF BLOODSTREAM AND OTHER INFECTIONS

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Enterococci emerged in the early '80's as leading nosocomial pathogens. The appearance of vancomycin resistance among enterococcal isolates in the late '80's effectively eliminated the last therapeutic option for treating many of these infections. *Enterococcus faecalis* causes 75 - 90% of all enterococcal infections. With a view toward deriving new antimicrobial therapies, we undertook several studies on the pathogenesis of *E. faecalis* infection. We observed that among *E. faecalis* bacteremia isolates, a disproportionate number of those responsible for a horizontally transmitted hospital ward outbreak were of a cytolytic phenotype. Isogenic strains specifically defective in cytolysin expression were constructed and compared in a bacteremia model as well as in an endophthalmitis model. In both systems, the cytolysin was observed to make a significant contribution to virulence, contributing to the rate of appearance of enterococci in the bloodstream in the former model, and to direct tissue toxicity in the latter model. We characterized the cytolysin determinant by nucleotide sequence, transposon and site specific mutagenesis, intracellular and extracellular complementation, and by biochemical analyses. The cytolysin was observed to consist of two posttranslationally modified oligopeptide subunits distantly related to the lantibiotic family. Both structural subunits were shown to possess the lanthionine residues that are the hallmark of this family, but otherwise bear little structural or functional resemblance to members of this class. Following posttranslational modification, both cytolysin structural subunits undergo two sequential but independent proteolytic processing steps, one prior to or concomitant with secretion, and the second extracellularly. Following these modifications, the cytolysin is capable of effecting lysis of charged liposomes, bacterial cells, or eukaryotic target cells.

# VIRULENCE IN STREPTOCOCCUS PYOGENES

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*Streptococcus pyogenes* (group A streptococcus; GAS) is an important human pathogen that causes many different types of disease ranging from pharyngitis ("strep throat") and pyoderma to severe invasive diseases like myositis, fasciitis and streptococcal toxic shock syndrome. Unlike many other bacterial pathogens, it appears that the same strain of GAS can cause many or all of the above syndromes. We have been using several approaches to learn more about virulence factors in this versatile organism.

Because several presumptive virulence factors require the transcriptional activator Mga (multiple gene regulator of group A streptococcus) for expression, we have been investigating the mechanism of this regulation. Recently, we found that *mga* is expressed best in log phase and is turned off in stationary phase, unlike many secreted virulence factors in e.g. *Staphylococcus aureus*. Because some genes for other presumptive virulence factors that are not *mga*-regulated are also turned off in stationary phase, we suggest that at least one other global regulator controls this type of growth phase regulation.

We have developed 2 mouse models that mimic human respiratory GAS disease by using the GAS strain identified as a natural mouse pathogen. Using "knock-out" mutations in this GAS strain, we were surprised to find that the M and M-like proteins, long considered the major virulence factors of GAS, have no effect, while production of the hyaluronic acid capsule is essential to cause disease in these models.

## ISOLATION OF *BACTEROIDES FRAGILIS* MUTANTS WITH ALTERED GROWTH IN *in vivo* MODEL SYSTEMS.

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*Bacteroides fragilis* is a remarkably aero-tolerant, but obligately anaerobic Gram negative bacillus causing infections in man. The ability of pathogenic bacteria, in particular obligate anaerobes, to deal with the toxic products of oxygen metabolism is essential for their virulence. Several genetic systems to study virulence factors in this organism have recently been developed. In addition, the growth of *B.fragilis* in model systems consisting of mouse embryo fibroblasts (Monika) or Chinese Hamster Ovary (CHO) cells growing as monolayers in normal atmosphere supplemented with 5%$CO_2$, is being used to analyze some of the bacterial-host cell interactions required for *in vivo* growth and pathogenicity.

Using a derivative of Tn4400, a transposon originally isolated from *B.fragilis* plasmid pBFTM10, to mutagenize the chromosome of *B.fragilis* TM4000; several thousand independent mutants have been analyzed for altered growth in Monika and/or CHO cell cultures. Candidates which fail to grow or grow more slowly in the model systems include mutants with additional nutritional requirements (amino acids, guanosine), as well as strains that grow normally *in vitro* under anaerobic conditions. Many of the monolayer growth deficient (MGD) strains are also defective for growth in the rat granuloma pouch model system. The DNA sequence in the vicinity of the inserted transposon has been determined for some of these mutants. Insertions into a gene with strong homology to the *mox*R gene found in methanol utilizing bacteria and in most bacterial pathogens have been isolated twice. In addition, these mutants show enhanced $O_2$ sensitivity and are deficient for growth in the rat pouch.

Other genes identified in the MGD mutants include a homolog to rubredoxin (83% similarity), an Fe-S protein known to be involved in $O_2$ detoxification, located in the vicinity of a potential histidine kinase. Candidates with altered binding to tissue culture cells, altered survival in $O_2$ or $H_2O_2$, have also been isolated but their DNA sequence reveals ORF's with matches to hypothetical proteins in other bacteria, or with no significant matches in the data base. Complementation experiments with cloned libraries of *B.fragilis* chromosomal DNA are being used to identify the gene(s) inactivated by the Tn4400 insertion events.

In an effort to identify *B.fragilis* genes that are specifically activated when the bacteria are growing *in vivo* , we have adapted the lox/cre system for use in *B.fragilis* and have begun to isolate promoter fragments which are expressed under specific environmental conditions; some of the challenges include shift from anaerobic to aerobic conditions, treating anaerobic cultures with $H_2O_2$, switch from *in vitro* to tissue culture or rat pouch conditions.

# ATTACHMENT OF *Streptococcus pneumoniae* TO HUMAN BRONCHIAL EPITHELIAL CELLS (BEAS-2B)

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**Adherence to epithelial cells of the respiratory tract is a prerequisite for colonization and infection by *Streptococcus pneumoniae*. Pneumococcal adherence to alveolar epithelial cells and nasopharyngeal epithelial cells has been well characterized. However, the interaction of *S. pneumoniae* with bronchial epithelial cells has not been studied. We have now shown that pneumococci bind specifically to a human bronchial epithelial cell line (BEAS-2B cells). Pneumococci adhered to BEAS-2B cells in a time and dose-dependent manner. These results suggest that the bronchial epithelium may serve as an additional site of attachment for pneumococci and demonstrate the potential utility of the BEAS-2B cell line for studying mechanisms of pneumococcal infection.**

# THE IL2/IL2R SYSTEM AS A T AND NK CELL ACTIVATION CLOCK

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A mathematical theory is presented describing the populations of receptor subunits, IL2 secretion and binding, and their interactions on single T and NK cells. The primary stimulation of T cells results in a cytokine/receptor induction distinct from that of a secondary stimulation. Critical roles of the different receptor subunits are suggested -- the subunits act as parts of a "clock" which times and limits cell proliferation. Defects of this clock are discussed in light of the theory. Applications are made to general viral infection dynamics, and to specific HTLV--1 and HIV--1 infection phenomena, as well as IL2 chemotherapy protocol.

# STRUCTURE AND FUNCTIONAL ANALYSIS OF A VACCINIA VIRUS HOST RANGE GENE

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Vaccinia virus expresses a number of host range (hr) genes that control virus growth in distinctive cell types. In CHO cells a hr gene CP77 suppresses apoptosis induced by virus infection. In addition, CP77 allows virus life cycle proceed to late phase and infectious virions could be produced. Overexpression of bcl-2 gene also suppresses apoptosis of CHO cells infected by vaccinia virus. however, suppression of apoptosis did not rescue virus growth, suggesting hr genes have other activity besides apoptosis suppression.

CP77 gene was isolated before and the sequences did not reveal homology with any known genes except ankyrin repeats. To understand the mechanism of host restriction in CHO cells, we generated various mutations of CP77 gene by in vitro mutagenesis and subsequently constructed recombinant vaccinia virus containing CP77 mutants. Experiments are in progress now to determine the domains of CP77 protein important for its host range functions.

SPECIFIC FILAMENT TIP ASSOCIATED ANTIGENS OF *NOCARDIA ASTEROIDES* GUH-2 IN ASSOCIATION WITH γδ T LYMPHOCYTES ARE INVOLVED IN INVASION OF PULMONARY EPITHELIA FOLLOWED BY DISSEMINATION TO THE BRAIN AND KIDNEYS

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*N. asteroides* is a filamentous bacterium that causes either acute or chronic pulmonary infections in both normal and immunocompromised individuals. In humans, there is frequent dissemination from the lungs to the brain and kidneys. The filament tip of log phase cells of *N. asteroides* GUH-2 attaches to and penetrates the surface of a variety of endothelial and epithelial cells both in vitro and in vivo. Antibodies specific for a 43kDa protein and the glycolipid, cord factor (CF), bind to the filament tip of GUH-2 and prevent attachment to and invasion of cells.

Pulmonary γδ T cells were involved in protecting the lung from nocardiae and altering dissemination to other organs since knock out mice lacking these cells showed a differential response to GUH-2 as compared to C57/BL6 controls. Data suggested that γδ T cells interacted with CF on the filament tip of GUH-2 during the initial stages of infection because monoclonal antibody to CF blocked adherence and dissemination in control C57/BL6 mice but not in γδ T cell knock out animals. Thus, over a period of 3 hours to 5 days, 63.6% (14/22) of C57/BL6 mice infected IN with GUH-2 had nocardiae recoverable from the brain, whereas only 1 of these mice (1/22; 4.5%) had organisms in the kidneys and none had organisms in the blood. In contrast, γδ T cell knock out C57/BL6 mice infected IN with GUH-2 had 72.7% (16/22) dissemination to the kidneys; 40.9% (9/22) in the brain and 27.8% (5/18) positive blood cultures. No dissemination of nocardiae was observed in C57/BL6 mice infected with GUH-2 preincubated with monoclonal antibody to CF. It is not clear why γδ T cell knock out mice had increased dissemination to the kidneys as compared to the brain.

## THE HEMOPHORE-DEPENDANT HEME ACQUISITION SYSTEM OF SERRATIA MARCESCENS.

S.Bertin, S. Letoffé, J.M. Ghigo, R. Binet, P. Delepelaire, C. Wandersman.

Iron is an essential nutriment and its acquisition a virulence factor for most bacterial pathogens. In vertebrate host most of the intracellular iron is complexed with ferritin, or associated with porphyrin ring as heme or hemoproteins such as hemoglobin. The extracellular iron is bound to transferrin or lactoferrin. Bacteria have developed specific functions that allow them to acquire iron from these various host organism sources in particular from heme. The external membrane of Gram negative bacteria is impermeable to heme, but some of them possess specific heme acquisition system. Generally it involves a specific membrane receptor for heme or hemoprotein-heme complex. Heme translocation step to the periplasm through the external membrane is energy consuming and TonB dependent.

*Serratia marcencens*, an opportunist pathogen is also able to use free heme or heme from hemoglobin as unique iron or porphyrin source by an original way. This acquisition system depends on a 19kDa extracellular protein HasA secreted in condition of low iron environment witch possesses a high affinity for free heme, or heme bound to hemoglobin. HasA is secreted in one step by an ABC transporteur composed by two inner membrane protein, HasD an ATPase named ABC protein and HasE the membrane fusion protein, and one outer membrane component, HasF. This system was studied in *E. coli hemA*, a heme auxotroph unable to use exogenous heme compounds, by looking for the host and foreign components required to allow heme or hemoglobin utilisation. We identified a 92 kDa iron regulated outer membran protein, HasR, which on one hand enabled alone *E. coli hemA* to grow on heme or hemoglobin, and on the other hand when coexpressed with HasA greatly improved heme acquisition from hemoglobin. The heme translocation step through the outer membrane is TonB dependent. All the genes encoding this heme acquisition, except *hasF* encoding the outer membrane component of the HasA ABC transporteur, are clustered in the *has* operon and are iron regulated. Downstream to those genes we found *hasB*, an iron regulated gene, encoding HasB a membrane protein of 28kDa presenting homologies with the TonB family. It is a proline rich protein with an hydrophobic N-terminal segment suggesting an anchorage in the cytoplasmic membrane with a large periplasmic domain. HasB expressed in an *E. coli tonB* mutant does not restore any of the TonB phenotypes. More over HasB coexpressed with HasA and HasR does not enable *E. coli hemA tonB* to grow on heme or hemoglobin. A HasA-HasB fusion was constructed and purified as inclusion bodies. Antibodies direct against this chimera were raised in rabbits and used in western blotts to detect HasB. They revealed an iron regulated protein with an apparent molecular weight of 34kDa in *S. marcescens*. In a *S. marcescens* strain harbouring a plasmid expressing *hasB* constituvely, it was detected also in iron rich medium. However it was not present in *E. coli* harbouring the same plasmid suggesting that HasB is unstable in *E. coli*. Instability of TonB without cofactors ExbB and ExbD in *E. coli* led us to postulate that, similary, HasB is unstable in *E. coli* in the absence of some specific *S. marcescens* cofactor. The *hasB* gene is currently disrupted in *S. marcescens* to determine the TonB and HasB respective functions in heme uptake.

# OLIGOPEPTIDE PERMEASE ENCODED BY BOTH CHROMOSOMAL AND PLASMID LOCI IN *BORRELIA BURGDORFERI*

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The infectious agent of Lyme disease, *Borrelia burgdorferi*, adapts to different environments during the tick-mammal infection cycle. Bacterial oligopeptide permeases, five membrane-associated proteins belonging to the ABC transporter family, provide a mechanism for the uptake of small peptides, which not only provide nutrients but often serve as environmental signals that lead to a variety of adaptive responses. To initiate an investigation of the role of the *B. burgdorferi* oligopeptide permease in environmental sensing, we have identified a chromosomal locus that encodes homologs of all five components. An interesting feature of this transporter in *B. burgdorferi* is the presence of multiple copies of the gene encoding the peptide-binding component, OppA, with three at the chromosomal locus and two on plasmids. Plasmid-encoded OppA homologs in other bacteria serve as receptors for specific peptide pheromones, which initiate conjugation and transfer of plasmid. In vitro, all five *B. burgdorferi oppA* genes are expressed, although the levels and patterns of transcript differ among genes with respect to varying culture conditions.

We have undertaken both genetic and biochemical studies to characterize the components of oligopeptide permease and to identify their physiological roles in the natural infectious cycle of *B. burgdorferi*.

# *CANDIDA ALBICANS* GENES REGULATED BY *TUP1*, A REPRESSOR OF FILAMENTOUS GROWTH

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*TUP1* of *Candida albicans* is a transcriptional repressor that transmits environmental signals to its target genes. We have recently described a *tup1* deletion mutant that displays constitutive filamentous growth, presumably due to de-repression of filamentous growth-promoting genes (Science, July 4, 1997). We have isolated genes that are over-expressed in this *tup1* mutant strain of *C. albicans* relative to wild-type parental cells. These genes were assayed for expression using Northern blotting and slot blotting with RNAs from *TUP1*-depleted cells, wild-type cells, and wild-type cells treated with serum and other inducers of filamentous growth. Three genes induced by both serum and *TUP1*-depletion were chosen for further sequence analysis. Two of these genes are related to what appears to be a family of cell-wall resident proteins of unknown function. The third gene, when deleted, causes a specific reduction of filamentous growth on certain media, including YEPD and spider plates. Additional regulated genes will be discussed.

# EXPRESSION OF THE CYTOKINE GM-CSF IN TRANSGENIC MICE

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We have produced an acute phase expression vector based on the promoter and flanking regions of the human C-reactive protein (CRP) gene to enable genes of interest to be expressed in a controlled manner. Using this vector we have demonstrated controlled interleukin-inducible *in vitro* expression of the haemopoetic cytokine granulocyte macrophage colony stimulating factor (GM-CSF). GM-CSF controls the differentiation of bone marrow stem cells into granulocytes and monocytes.

Using this CRP/GM-CSF fusion vector we have produced transgenic mice which express GM-CSF. Increased levels of this cytokine should lead to increased numbers of activated granulocytes and macrophages, and may therefore predispose to an increased ability to combat certain pathogens. Analysis of the blood of these mice showed a marked increase in the number of leukocytes compared to non-transgenic controls. Experiments have also been carried out to examine the effects of LPS stimulation on GM-CSF expression.

The transgenic mice will be challenged with the bacterial pathogens *Streptococcus pneumoniae* and *Listeria monocytogenes* in order to determine their susceptibility to disease in comparison to non-transgenic littermates. These studies will help us to elucidate the role of GM-CSF in immunity to different types of pathogen.

Our work will be of value in the production of transgenic animals showing controlled expression of any gene of interest, and in improving understanding of host-pathogen interactions. In the longer term it may be valuable in the development of drug therapies for human diseases, and of disease resistant animal breeds.

## Mp1 Encodes An Abundant And Highly Antigenic Cell Wall Mannoprotein In Pathogenic Fungus *Penicillium Marneffei*

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The integrity of the fungal cell wall is critical for cell structure and survival in the natural environment. Mannoproteins are the one of the major structural components of fungal cell wall. Studies in the yeast *Saccharomyces cerevisiae* suggested that mannoproteins may contribute to a variety of diverse biological functions: determining cell shape, supporting cell growth and morphological change, serving a protective role, allowing sex agglutination, and limiting the porosity of the cell wall. *Penicillium marneffei*, a dimorphic fungus, is the most common fungal pathogen found in AIDS patients in Southeast Asia We have cloned a gene, MP1, that encodes a highly abundant antigenic cell wall mannoprotein from *P. marneffei*. It codes for a protein of 462 amino acid residues with a few sequence features that are present in several cell wall proteins, a serine/threonine-rich region, a signal peptide and a putative glycosylphosphatidylinositol attachment signal. Western blot analysis with specific anti-Mp1p antibody revealed that Mp1p had a predominant molecular weight of 58 kDa. It belongs to a group of cell surface proteins that can be readily removed from yeast cells by glucanase digestion. Immunoprecipitation and glycoprotein assay indicated that Mp1p was a highly abundant yeast glycoprotein. It had a high affinity for Concanavalin A, indicative of a mannoprotein. Using immunogold staining under electron microscopy, we demonstrated that Mp1p was a common protein found in the cell wall of yeast, hyphae and spores of *P. marneffei*. Ultrastructural analysis revealed that Mp1p was located throughout the entire thickness of *P. marneffei* cell wall. MP1 is the first cell wall mannoprotein gene identified from pathogenic fungi. We observed that infected patients developed high levels of antibody response against Mp1p, suggesting that this protein represents a good cell surface target for host immunity.

# THE MULTIPLE CATALYTIC ACTIVITIES OF THE PURIFIED, RECONSTITUTED *STAPHYLOCOCCUS AUREUS* TETA(K) PROTEIN.

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Gram-positive tetracycline (Tc) efflux proteins have recently been shown in our laboratory to catalyze monovalent cation/proton antiport, in which either cytoplasmic $Na^+$ or $K^+$ are exchanged for external protons(1). This activity is a physiologically important one, in pH homeostasis and $Na^+$ resistance of the chromosomally-encoded Tet(L) protein of *Bacillus subtilis*(2). Purified, reconstituted TetA(L) exhibits both Tc-$Me^{++}/H^+$ and $Na^+(K^+)/H^+$ antiport. Experiments in whole cells as well as in isolated membrane vesicles suggested that the related TetA(K) protein encoded by the *Staphylococcus aureus* plasmid pT181 was similarly multifunctional(3). In addition, several studies suggested that, as with the TetA(C) encoded by pBR322, TetA(K) could catalyze net $K^+$ uptake; this activity has been found in examples of the related but topologically distinct TetA(C) and TetA(K) but not in their closer respective homologs, TetA(B) and TetA(L), respectively(4,5).

Earlier investigators had ascribed the net $K^+$ uptake catalyzed by some Tet proteins to a leak that allowed $K^+$ flux in response to the transmembrane potential, positive out, developed across the cell membrane(4). Given the finding of monovalent cation/$H^+$ antiport as one of the catalytic modes of these proteins, we hypothesized that the net $K^+$ uptake could instead represent a catalytic mode in which cytoplasmic $Na^+$ or $K^+$ was exchanged for external $K^+$ in an electrogenic manner, e.g. $K^+/Na^+>1$, energized by the transmembrane potential. In the current studies, hexahistidine-tagged TetA(K) has been purified and reconstituted into proteoliposomes. The protein exhibits Tc-cobalt/$H^+$ antiport activity in response to an imposed pH gradient. To assess whether net $K^+$ uptake occurs as an electrogenic exchange or a leak, proteoliposomes were loaded with the lipophilic cation tetraphenylphosphonium ($TPP^+$) together with either $Na^+$, $K^+$, or choline. The proteoliposomes were diluted into $TPP^+$-free buffer containing $^{86}Rb^+$ (to monitor $K^+$ flux). Diffusion potential dependent uptake of $^{86}Rb^+$ was also dependent upon the presence of internal $K^+$ or $Na^+$, with $Na^+$ being a far superior substrate for the apparent exchange. Thus, the previously observed $K^+$ uptake capacity of some Tet proteins is likely to be an exchange mode of the monovalent cation antiport activity in which $K^+$ occupies the external $H^+$ site(s).

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A C3 BINDING PROTEIN IN *STREPTOCOCCUS PNEUMONIAE*

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The third component of complement, C3, mediates opsonization and phagocytosis of *Streptococcus pneumoniae* in the non-immune host. We therefore searched for pneumococcal surface proteins that might interact with C3. Incubation of Western blots of lysates and supernatants from exponentially growing pneumococci ( strain CP1200) with 2 μg/ml of biotinylated C3 detected a band of 90 kDa. Binding of C3 was non-opsonic, non-covalent, and independent of thiolester conformation. The identical protein was identified in both encapsulated and unencapsulated clinical isolates (serotypes 1, 3, 4, 14, 19F, and 23F). The 90 kDa protein was absent in lysates and supernatants from a 23F strain that failed to cause otitis media in a chinchilla model. Serum from patients recovering from acute pneumococcal infection contained IgG antibodies to the 90 kDa protein. After 4 hours' incubation with a monolayer of type II pulmonary epithelial cells (A549), less than 1 μg of purified 90 kDa protein induced the release of 761 pg/ml IL-8, equivalent to a stimulus of 0.5-1.0 unit of IL-1. FITC-labeled C3 bound to the pneumococcal surface in the absence of other complement proteins. The first 400 amino acids of the cloned gene product displayed no substantial homology to other pneumococcal proteins in the database, including PspA, PsaA, and pneumolysin. Thus a 90 kDa C3-binding protein from *Streptococcus pneumoniae* is surface-borne, structurally conserved in a variety of clinical and laboratory isolates, and implicated in virulence.

# FUNCTIONAL ANALYSIS OF GLUCOSYLTRANSFERASES IN STREPTOCCUS MUTANS

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Streptococcus mutans glucosyltransferases (GTFs; GtfB, C and D) are enzymes responsible for the synthesis of water soluble and insoluble glucan polymers from glucose. We have identified previously an N-terminal conserved region of 19 aa (Gtf-p1, 409 to 427 of GtfB and 435 to 453 of GtfC) which is located 19 residues N-terminal of the active region containing the catalytic aspartic acids, Asp451 and Asp478. We initiated a genetic approach, using different strategies of mutagenesis, to analyze the functional role of Gtf-p1 in enzymatic activities of GtfB and GtfC, which share identical primary structure around this region but are functionally distinct entities. In-frame insertion or deletion within this region abolished the enzymatic activities of both GtfB and gtfC, but their glucan binding affinity was unaffected. Substitution of several residues in combination by random mutagenesis resulted in mutants exhibiting detectable sucrase and decreased glucan synthesizing activity. Site-directed mutagenesis was subsequently carried out to confirm the role of several amino acids in this region of both enzymes. Amino acid substitutions of carboxylic acid residues turned out to be more critical for activity than at other positions of this region. Furthermore, the putative catalytic residues may play distinct roles in these closely related enzymes. These results indicated that, analogous to other transferases and glucosidases, S. mutans GTFs require additional carboxylic acids for enzymatic activity.

A MURINE MODEL FOR INFECTIOUS COLITIS.

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Inflammatory bowel disease (IBD) comprises a group of disorders with no known etiology. The most well known forms are Crohn's disease and ulcerative colitis. Recently, it has been found that gene-targeted mutant mice including T cell receptor $\alpha$ and $\beta$ (TCR $\alpha$, TCR $\beta$) chain knockouts develop spontaneous IBD lesions. Initially, cultures for murine pathogens were negative. Subsequently, a new pathogen was discovered - *Helicobacter hepaticus*. The TCR mutant mice were retested and found to be *H. hepaticus* positive. Germfree TCR $\alpha$ mutant mice do not develop disease. In studies with immunodeficient mice, *H. hepaticus* has been associated with the development of colitis as well as chronic hepatitis and hepatocellular neoplasia.

T cell receptor mutant mice have been rederived to be *Helicobacter*-free in our laboratory. At 4 months of age, mice with enteric microflora have no clinical or histological signs of IBD. At 1 year, a few mice develop lesions. Infection with *Citrobacter rodentium*, another murine pathogen, results in severe IBD. An experimental infection study with *H. hepaticus* is in progress.

**Immunoepidemiologic Investigation Of Japanese Encephalitis Viral Infection In Several Ecologic Settings In Taiwan And Jev Immunity In Mice**

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Japanese Encephalitis Virus(JEV) is a zoonotic infection with high case fatality rate. We conducted an immunoepidemiologic study among different ecological communities and elementary schools by JEV-ELISA-IgM. Our data found that: (1). Residents living in Hopin Li of Yuanlin Township without pig farm and rice field had highest recent JEV infection, with 7.76%(9/116) JEV-ELISA-IgM seroincidence; residents in Lunya Li of Yuanlin Township both pig farms and rice fields showed 3.39%(2/59) JEV-IgM seroincidence; residents im Yuanpi Li of Homei Township had rice fields but without pig farm showed 2.9%(8/276) JEV-IgM seroincidence; (2). JEV seroincidence rate was increased in grade 4 or 5 elementary school children; (3). JEV Nakayama strain used as ELISA-IgM antigen was more sensitivity than JaGAr-01 strain among school children. Further immunologic assessments are under progress to investigate the immunologic problems of vaccine by mouse model. Our preliminary data showed that different route of injection may induce different level viremia as well as immunity in various lymphoid tissues.

# INTEGRIN BINDING BY LYME DISEASE SPIROCHETES

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The Lyme disease agent, *Borrelia burgdorferi (sensu lato)*, expresses at least two distinct pathways that may contribute to binding to mammalian cells. The spirochetes recognize heparin- and heparan-sulfate proteoglycans, a pathway that mediates attachment to extracellular matrix as well as to cell surfaces. In addition, *B. burgdorferi* is able to bind to one or more integrins, which are expressed on the surfaces of mammalian cells, but not in the extracellular matrix. We have characterized *B. burgdorferi* attachment to the widespread integrins $\alpha_v\beta_3$ (the vitronectin receptor) and $\alpha5\beta_1$ (the fibronectin receptor), and to the platelet-specific integrin $\alpha_{IIb}\beta_3$. While binding to platelets appears to be mediated primarily by $\alpha_{IIb}\beta_3$, the proteoglycan and the integrin pathways both contribute to binding to epithelial cells. Since different *Borrelia* strains appear to exhibit distinct integrin and proteoglycan recognition profiles, it is possible that the tissues infected, and the manifestations of disease, might be affected by the specific adhesion properties of the infecting spirochetes.

In order to clone *B. burgdorferi* integrin ligand(s), a library of genomic DNA that allows expression of foreign sequences as fusions with gene III on the surface of filamentous phage was constructed. Phage clones that bind specifically to $\alpha_{IIb}\beta_3$ were analyzed. One candidate, represented by two independent isolates that contain overlapping fragments of the *B. burgdorferi* genome, binds to both $\alpha_{IIb}\beta_3$ and to $\alpha_v\beta_3$. The larger fragment encodes one continuous open reading frame of 237 amino acids that is in frame with the phage gene III product, and represents a portion of an outer membrane protein. Further analysis of this clone is underway. For example, fusions of this *B. burgdorferi* protein to MBP have been shown to bind integrins, demonstrating that the activity observed is not simply an artifact of the junction of phage-*Borrelia* sequences. Identification of the *B. burgdorferi* integrin ligand(s) will eventually allow us to assess the role of binding to particular integrins in the pathogenesis of Lyme disease, and may provide unique insight into integrin biology.

BI-COMPONENT LEUKOTOXINS FROM *STAPHYLOCOCCUS AUREUS.*

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*S. aureus* secretes a protein family (Leukocidin, γ-hemolysin) of about 35 kDa which are classified in S components : LukS-PV, HlgA, HlgC, with 63 to 75 % identity and F components : LukF-PV, HlgB with 70 % identity. The associations of a S with a F component constitute bi-component leukotoxins whose main targets are the polymorphonuclear neutrophils (PMNs) and the monocytes ; the pair HlgA/HlgB is also haemolytic and toxic for some lymphocytes.

The first event of the leukotoxin activity is the binding of the S component followed by the binding of the F component. The binding site appears during the differentiation of the PMNs at the metamyelocyte stage. A $K_D$ of 0.13 nM was determined on human PMNs and 0.04 nM on monocytes for LukS-PV by flow cytometry using a fluorescein-labelled active cystein mutant. PMNs bind three times more LukS-PV molecules than monocytes. No binding of LukS-PV were observed on lymphocytes. The study of the functional relationships between mutated leukotoxins and the target cells have shown that the binding of the F component necessitates the integrity of a predicted β-sheet conserved in all the S components.

The leukotoxic activity which appears after the binding of the two components is caused by two successive events modifying the membrane permeability of the target cells. These events were studied by flow cytometry and spectrofluorometry using molecular fluorescent probes. The first event is the opening of $Ca^{2+}$ channels in the absence as in the presence of extracellular $Ca^{2+}$, which are inhibited by $Ca^{2+}$ channels blockers Furthermore, in $Ca^{2+}$ medium, $Ca^{2+}$ release-activated $Ca^{2+}$ channels which can be inhibited by treatment with thapsigargin, TMB8, tyrphostin A9 and $La^{3+}$ ions are opened. The consecutive increase of the intracellular $Ca^{2+}$ activates the PMNs and induces the exocytosis of their granule content. The second event is the constitution, after insertion and oligomerisation of the two components into the membrane, of a transmembrane pore, revealed by ethidium influx. This pore (r=1.2nm) is impermeant to divalent cations but seems to be permeable to $Na^+$ and $K^+$. Conversely to α-toxin, another pore-forming toxin from *S. aureus*, the bi-component leukotoxins are unable to provoke the apoptosis of the target cells.

In conclusion, the leukotoxins induce an uncompetent immune response of host defense cells which may contribute to tissue damage, and the death of the cells by necrosis. These inflamatory events may be of fundamental importance in the pathogenesis of a variety of clinical disorders implicating *S. aureus*. Besides their clinical implications, these bi-component leukotoxins constitute valuable tools for the study of protein-protein and protein-plasmic membrane relationships.

# ALTERED PATTERNS OF SYNTHESIS AND PROCESSING OF EXTRACELLULAR PROTEASES IN AN AVIRULENT SPONTANEOUS MUTANT OF *P.GINGIVALIS* W50.

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The extracellular proteases of *Porphyromonas gingivalis* are considered to be important factors in the virulence of this organism. A non-pigmenting mutant of *P.gingivalis* W50, W50/Bel, has been shown to be avirulent in animal models and to produce significantly less arginine-specific protease activity than the parent strain. Three proteases are present in the culture supernatant of *P.gingivalis* W50: RI (a heterodimer consisting of a ~55kDa protease α-chain and a ~58 kDa adhesin β-chain), RIA (consisting of the ~55 kDa protease α-chain) and RIB (70-80 kDa, in which the α-chain is covalently modified with carbohydrate). All three proteases are derived from *prpRI* which encodes a 185.7 kDa polyprotein that is organised into distinct domains (pro, α, β and γ).

The aim of the present investigation was to determine the biochemical nature of the arginine-specific proteases produced by the avirulent W50/Bel strain. Extracellular proteases were purified by a combination of ammonium sulphate precipitation, differential detergent solubilisation, gel filtration, affinity and ion-exchange chromatographies, and the resultant pure preparations were subjected to SDS-PAGE, N-terminal amino acid sequencing and enzyme kinetic studies.

Significant differences were observed between the proteases of *P.gingivalis* W50/Bel and W50. We were unable to detect RIA and RIB and only very low concentrations of RI in the culture supernatant of W50/Bel. Furthermore, unlike the parent strain enzyme, RI from W50/Bel was composed of three polypeptide chains including one derived from the γ region of the PrpRI polyprotein precursor. However, the majority of the arginine-specific protease activity in W50/Bel was derived from a second gene *prR2*, and consisted of two enzymes (RIIA/Be and RIIB/Be) both of which contained two peptide chains: a ~55 kDa chain corresponding to the protease domain and a ~26 kDa chain corresponding to the N-terminal propeptide domain of the initial PrRII precursor. RIIB/Be lacked the carbohydrate modifications seen in RIB. These data indicate that the reduced level of protease activity in W50/Bel may reflect both reduced synthesis of *prpRI* enzymes, an altered post-translational modification pathway and aberrant processing of protease gene translation products in this mutant.

**A Sucrose Sensitivity Marker For Introduction Of Unmarked Mutations Into *Helicobacter Pylori***

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Research on *Helicobacter pylori* has been hindered by the lack of useful genetic tools. Using the sacB gene of *Bacillus subtilis*, we developed a sucrose-based counterselection system that allows introduction of unmarked mutations in *H.pylori*. A kan-sacB cassette, consisting of the sacB gene expressed from the *H.pylori* flagellin promoter and a kanamycin resistance module, was introduced by homologous recombination into a target *H.pylori* gene. The resultant strains were sucrose sensitive and kanamycin resistant. Following transformation with a mutated allele, growth in sucrose-containing medium allowed the selection of strains that had lost the kan-sacB module and had integrated the unmarked allele. We have used this cassette to perform a site-directed modification of two histidine residues encoded by the vacA gene in a two-step procedure. This system should prove useful in the site-directed mutagenesis of *H.pylori* genes, including creation of nonpolar mutations in putative virulence factors, modification of various promoter sequences, and introduction of high-affinity antibody tag sequences to facilitate biochemical studies and immunohistochemical analysis of secreted proteins.

# REGULATION OF IRON METABOLISM IN MYCOBACTERIA: LINKAGE TO OXIDATIVE STRESS RESPONSE AND DRUG RESISTANCE

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Iron stress and oxidative stress are closely interconnected in aerobic organisms. Cellular toxicity can occur especially via Fenton-dependent oxidative chemistry. Therefore, microorganisms produce protection systems to counteract the potentially lethal iron/reactive oxygen species partnership. Iron uptake and oxidative stress bacterial genes have been shown to be under a tight and complex regulation. In mycobacteria, IdeR, a homologue of the *Corynebacterium diphtheriae* repressor protein DtxR, regulates siderophore biosynthesis. Similarly to *Escherichia coli fur* mutants, *Mycobacterium smegmatis ideR* mutants showed altered response to oxidative stress. IdeR-defective *M. smegmatis* were more sensitive to reactive oxygen species generators and had reduced KatG and SodA activities. *ideR* mutants were also more susceptible to the antitubercular agent isoniazid. However, they were not more sensitive to cumene hydroperoxide and expressed wild-type levels of AhpC, suggesting that the increased susceptibility to isoniazid was alkyl hydroperoxidase-independent. The residual level of KatG was presumably sufficient to induce the peroxidatic conversion of the prodrug to its active form. In addition, the low level of SodA activity may have a role in the increased susceptibility to isoniazid. The role of superoxide radicals in isoniazid activation and toxicity is under investigation.

# GENETICAL REARRANGEMENTS IN THE PATHOGENICITY LOCUS OF *CLOSTRIDIUM DIFFICILE* STRAIN 8864 AFFECTING REGULATION OF TOXIN EXPRESSION

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We investigated genetical changes in the genes tcdA-E of the Pathogenicity Locus (PaLoc) of C. difficile 8864, a strain reported to be as toxin A (TcdA) negative but jet pathogenic. Overlapping PCR reactions were performed and two rearrangements were observed. A deletion of 5.9 kb covers two thirds of the tcdA gene and reaches out into the downstream tcdC gene, and an insertion of 1.1 kb upstream of the tcdA gene. With the aid of a novel monoclonal antibody, which detected the truncated recombinant TcdA-8864 molecule, culture supernatants of strain 8864 were searched for TcdA. However, the TcdA-8864 protein remained undetectable, while TcdB-8864 production was exceptionally high. Trying to explain the lack of TcdA-8864 production a transcription analysis was performed, which demonstrated that the two changes affected tcdA-gene transcription. The upstream insertion blocks monocistronic transcription of the tcdA gene. The deletion provokes readthrough transcription of tcdA into its downstream gene tcdC and vice versa. In sum, this abolished transcription of tcdA effectively. These data strengthen our regulatory model of the genes tcdA-E of the PaLoc (Braun et al, 1996; Hundsberger et al. 1997) of all C. difficile strains assigning positive regulatory function to tcdD and a negative to tcdC. The pathogenic potential of the strain will be discussed in relation to the genetical changes that we monitored.

Braun et al. (1996) Gene 181: 29-38
Hundsberger et al (1997) Eur. J. Biochem. 244: 735-742

USE OF Tn*917* TO GENERATE INSERTION MUTATIONS IN THE GROUP A STREPTOCOCCUS

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The *Enterococcus faecalis* transposon Tn*917* is functional in a broad range of bacteria, including both gram-positive and gram-negative species. We cloned Tn*917*-LTV3, a derivative carrying a promoterless *lacZ* gene, into the thermosensitive shuttle replicon pG$^+$host4 and assayed for chromosomal insertions in group A streptococcus (GAS). Tn*917* transposed into the GAS chromosome at a frequency of $2.8 \pm 3.2 \times 10^{-5}$ per colony forming units. Transposition products were predominantly simple insertions and no target site preference was detectable. Some transcriptional fusions were identified in which the promoterless *lacZ* of the transposon appeared to be expressed from an external promoter.

TRANSPOSON INSERTION IN THE *CYD* OPERON OF *BRUCELLA ABORTUS* ATTENUATES INTRACELLULAR SURVIVAL.

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Exposure of *Brucella abortus* to reduced pH environments, which mimic the intracellular environments in which it survives, has been shown to alter protein expression. Identification of the genes mediating growth and survival under reduced pH was attempted by a number of methods including screening a Tn5 mutant bank of *B. abortus* for growth at reduced pH (Å5.3). Eight mutants out of 1000 screened using this protocol were identified by their lack of growth and all were interrupted in a locus with homology to *E. coli cyd*AB, encoding the alternative terminal oxidase of the electron transport chain. *E. coli* cytochrome d (Cyd) oxidase is maximally expressed in environments with reduced levels of $O_2$, and is noted for its [$Na^+$] pump activity coupled to electron transport rather than [$H^+$] pumping. Changes in cytochrome content of *Brucella* consistent with a switch to cytochrome d oxidase were previously observed during late-log growth phase. The *B. abortus cyd*AB-like mutants were shown to be growth deficient within murine and bovine macrophages, and were rapidly eliminated from the spleens of infected mice. These mutants also lack an adaptive acid tolerance response, but it is not clear whether this is distinct from their overall acid sensitivity. In vitro studies also indicate an extreme sensitivity of the mutants to $H_2O_2$. This phenotype may be caused by the intracellular buildup of oxidative metabolites resulting from the block in electron transport. Presumably, Cyd oxidase provides a selective advantage within acidic cellular vacuoles, in which growth may be restricted by inhibition of proton pumping or limitation of intra-vacuolar oxygen. Experiments in progress are designed to confirm the genetic basis for the observed phenotype, as well as determining the intracellular conditions responsible for the attenuated survival of the *cyd* mutants.

**GENERATION OF ANTIBODIES WITH ADRENERGIC STIMULATING ACTIVITY IN CRONIC CHAGAS HEART DISEASE.**

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We have previously suggested that antibodies directed against the ribosomal P0 protein of *Trypanosoma cruzi* cross-react with the β1 adrenergic receptor (β1AR) (Ferrari *et al*, J. Exp. Med., 1995, 182: 59-65 and Elies *et al*, J. Immunol., 1996, 157: 4230-4211). The target of this cross-reaction was mapped to a region possessing a short stretch of negatively charged residues present in the C-terminal end of the parasite protein (AESEE) and in the second extracellular loop of the β1AR (AESDE).

In this report, we analyze the functional autoreactive properties of antibodies directed against *T. cruzi* ribosomal P1 and P2 proteins in chronic Chagas heart disease (cChHD). These antibodies, known as anti-P antibodies, present in sera from patients with cChHD recognize peptide R13, EEEDDDMGFGLFD, which encompasses the C-terminal region of the *T. cruzi* ribosomal P1 and P2 proteins. This peptide shares homology with the C-terminal region (peptide H13 EESDDDMGFGLFD) of the human ribosomal P proteins, which is in turn the target of anti-P auto antibodies in systemic lupus erythematosus (SLE), and with the acidic epitope, of the β1AR. Anti-P antibodies from chagasic patients showed a marked preference for recombinant parasite ribosomal P proteins and peptides, whereas anti-P auto antibodies from SLE reacted with human and parasite ribosomal P proteins and peptides to the same extent. A semi quantitative estimation of the binding of cChHD anti-P antibodies to R13 and H13 using biosensor technology indicated that the average affinity constant was about 5 times higher for R13 than for H13. Competitive enzyme immunoassays demonstrated that cChHD anti-P antibodies bind to the acidic portions of peptide H13, as well as to peptide H26R, encompassing the second extracellular loop of the β1AR. Anti-P antibodies isolated from cChHD patients exert a positive chronotropic effect in vitro on cardiomyocytes from neonatal rats, which resembles closely that of anti-β1AR antibodies isolated from the same patient. In contrast, SLE anti-P auto antibodies have no functional effect. These results suggest that the adrenergic stimulating activity of anti-P antibodies may be implicated in the pathogenesis of myocardial impairments observed in cChHD and may explain the fact that immunization of mice with the ribosomal P2 protein of *T. cruzi* induces alterations of the eletrocardiogram (Lopez Bergami *et al*, FEMS Immunol. Med. Microbiol., 1997, 18: 75-85).

## EVIDENCE FOR A β-GLUCOSIDASE ACTIVITY IN *HISTOPLASMA CAPSULATUM.*

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Enzymes with glucosidase activity are produced by a number of bacteria and fungi, including the human pathogens *Candida albicans* and *Coccidioides immitis*, and several potential roles have been hypothesized, including nutrient acquisition in particular environments and cell wall remodeling. The nucleotide sequence of the gene encoding the H antigen of the fungus *Histoplasma capsulatum*, a secreted glycoprotein and a major immunogen of this organism, reveals homology of the predicted protein sequence with that of some other fungal β-glucosidases. We have examined *H. capsulatum* for β-glucosidase production and made preliminary examination of this enzyme activity as a function of the H antigen. Hydrolysis of the chromogenic β-glucosidase substrate p-nitrophenol β-D-glucopyranoside (PNPG) was used as an assay for enzyme activity in culture supernatants concentrated by size exclusion spin columns. Conditions were optimized for microtiter plate assay of PNPG hydrolysis in the supernatants, including identification of artifactual reduction of enzyme activity due to a medium component. Examination of several strains of *H. capsulatum* demonstrated production of secreted β-glucosidase activity, with preliminary indication of strain variation in the level of production. PNPG hydrolysis by the culture supernatants was inhibited by castanospermine and 1-deoxynojirimycin, both competitive inhibitors of glycosidases, consistent with measurement of appropriate enzyme activity by our colorimetric reaction.

Additionally, a substrate gel electrophoresis assay of concentrated supernatants, using non-denaturing conditions and detection of PNPG hydrolysis in the gel after electrophoretic separation, indicated the presence of β-glucosidase activity in a single broad band of about 120 kD. The migration of this band of enzyme activity matches that of the native H antigen, as visualized by immunoblotting with polyclonal rabbit antiserum raised against the product of a recombinant H antigen gene expressed in *E. coli* (kindly provided by Dr. George Deepe). The bacterially expressed recombinant H antigen gene product, which migrates more rapidly than native *H. capsulatum* H antigen and presumably lacks glycosylation, does not show β-glucosidase activity in any of our assays. Efforts are in progress to determine whether the native H antigen of *H. capsulatum* is responsible for the β-glucosidase activity we have observed in culture supernatants, and to identify the possible significance and biological role of this enzyme.

# HIGH VOLTAGE ELECTRON MICROSCOPIC ANALYSIS OF THE INTRACELLULAR LOCALIZATION OF *MYCOBACTERIUM TUBERCULOSIS* WITHIN MACROPHAGES

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Recent studies using transmission electron microscopy (TEM) suggest that virulent H37Rv *Mycobacterium tuberculosis* (Mtb) are capable of escaping from membrane bound vacuoles to reside free within the cytoplasm of J774.16 macrophages. This extravacuolar phenotype is rarely seen in avirulent *M. bovis* BCG. However, the intracellular localization of Mtb after phagocytosis by macrophages is still a matter of debate. Conventional TEM (CTEM) techniques have been inconclusive in determining whether Mtb that appear to have escaped from their phagosomal compartments are free within the host cell cytoplasm or surrounded by a tightly apposed phagosomal membrane. We have used High Voltage Electron Microscopy (HVEM) on 0.25 micron thick specimens to further examine this apparent vacuolar escape phenotype. The tilt-rotation features of the HVEM specimen stage allowed us to perform stereo-image analysis of the intracellular localization of the bacteria. Stereo-imaging using the HVEM has proven extremely useful because it produces a three-dimensional image of the specimen that is easy to record and view, a distinct advantage over the two-dimensional images produced by CTEM. This three-dimensional information has allowed us to better differentiate between extravacuolar bacteria and those enclosed within tightly apposed membrane structures. Using HVEM stereo-image analysis, 74% of virulent H37Rv were determined to be localized within the cytoplasm of macrophages, and not surrounded by contiguous membrane structures, at 2 days post-infection. In contrast, less than 10% of the avirulent *M. bovis* BCG were determined to be extravacuolar at 2 days post-infection. Ultrastructural evaluation of the intracellular localization of Mtb may provide insight into this important pathogen's ability to evade the bactericidal mechanisms of macrophages.

# FUNCTIONAL ANALYSIS OF MYCOBACTERIAL SIGMA FACTORS A AND B

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The interaction between a pathogen and its host induces a response in both entities that is mediated, in whole or in part, by changes in gene expression. The main mechanism regulating gene expression in bacteria is transcriptional control, that can be exerted at the level of RNA polymerase sigma ($\sigma$) subunit mediated specific promoter recognition. Bacteria have a major, essential, $\sigma$ factor, required for the transcription of constitutively expressed genes and produce other alternate $\sigma$ factors that can replace the housekeeping $\sigma$, altering the specificity of the RNA polymerase. There are several examples of bacterial pathogens that regulate the expression of virulence genes in that way.

We have previously identified genes *sigA* and *sigB* encoding $\sigma$ factors in several mycobacterial species, including *M. tuberculosis* (Mtb) and *M. leprae*(Mlp). Because of the stronger similarity between *sigA* and *hrdB*, the major $\sigma$ from *Streptomyces coelicolor*, we speculated that *sigA* is the major $\sigma$ in mycobacteria while *sigB* could encode an alternate $\sigma$. Later *sigA* was identified as a virulence factor in *M. bovis* (Mbo). After infecting guinea pigs with an avirulent strain of Mbo transformed with a library of virulent Mbo DNA, Collins *et al* isolated a plasmid containing *sigA* that restored virulence to the attenuated strain. The attenuated strain was found to contain a point mutation in *sigA* (defining the allele that we call *sigA**) producing a single amino acid change near the carboxy end of the protein.

We have now obtained experimental evidence that *sigA* is an essential gene. Therefore, $\sigma^A$ is the housekeeping $\sigma$ in *M. smegmatis* (Msm), and presumably in other mycobacteria. The *sigA** mutation then should not inactivate $\sigma^A$ and we have found, accordingly, that the protein $\sigma^{A*}$ (purified from an *E. coli* overproducing strain) is still active in "in vitro" transcription assays. To study the physiological consequences of the *sigA** mutation we have obtained isogenic Msm strains that differ only in the *sigA** mutation. The *sigA** mutant does not show altered growth rate in liquid. However, the *sigA** strain is more sensitive to reducing agents, superoxide generators and some antibiotics.

The gene *sigB*, on the other hand, has been found to be dispensable in Msm, as we have been able to inactivate it by allelic replacement. The Msm *sigB*$^-$ strain does not show an altered growth rate in liquid when compared with the mc$^2$155 parental strain. However it is more sensitive to reducing agents, superoxide generators, $H_2O_2$ and Azide, but more resistant to isoniazid (INH), the front line antibiotic against Tb. Since resistance to INH is often associated with loss of catalase-peroxidase (KatG) activity, a positive role of $\sigma^B$ in KatG regulation is being investigated.

# ADHESION AND PENETRATION OF ORAL EPITHELIAL CELLS BY PERIODONTAL PATHOGENS

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The oral cavity presents one of the most complex microflora in nature. More than 90% of the periodontal diseases are caused by bacterial infections. So far, most of the studies are limited to *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Treponema denticola*, recognized as etiologic agents for periodontitis. One common feature of these three species is their capability of penetrating epithelial cells, a characteristic important for the bacteria either to cross the epithelium and underlying connective tissues to cause infections, or to evade host immune defense. In this study, we surveyed tissue adhesion and penetration activities of additional periopathogens, *Campylobacter curvus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, and *Prevotella intermedia*, using primary cultures of Normal Human Oral Keratinocytes (NHOK). We found that *F. nucleatum* penetrated NHOK in a "zipping" manner while *P. intermedia* adhered to NHOK in a similar way as EPEC to the epithelial cells, with "pedestal" structures.

## Anthrax Lethal Factor Hydrolysis Of Synthetic Peptides

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The lethal factor (LF) protein of *Bacillus anthracis* lethal toxin mediates macrophage cytolysis and death of the host during anthrax infections. LF contains a thermolysin-like active-site and zinc-binding consensus motif (-H-E-x-x-H-) required for zinc-coordination and cytolytic activities. LF is hypothesized to act as a Zn++-metalloprotease on specific protein targets found in the cytoplasm of macrophages. However, no direct proteolytic activities, or identification of a macrophage target, has yet been described for this toxin. We now demonstrate that various synthetic peptides are hydrolyzed by purified LF in vitro. After incubation with LF, hydrolysis of peptide substrates and formation of cleavage products were monitored by RP-HPLC. Mass spectroscopy and peptide sequencing of isolated cleavage products indicates that LF seems to prefer proline-containing substrates. Substitution mutations within the consensus active-site residues completely inhibit all catalytic functions, as does addition of the zinc chelators 1,10-phenanthroline or EDTA to in vitro reactions. Certain amino acid hydroxamates were also found to inhibit in vitro proteolysis. In contrast, metalloprotease inhibitors Bestatin and Lysine CMK previously shown to block LF activity on macrophages, did not inhibit the cleavage of peptide substrates. These data provide the first direct evidence that LF may act as an endopeptidase. Knowledge of LF recognition/cleavage motifs from these studies may be useful in efforts to identify pertinent cellular targets.

# THE DIPEPTIDE REPEAT REGION OF THE FIGRINOGEN-BINDING PROTEIN (CLUMPING FACTOR) IS REQUIRED FOR FUNCTIONAL EXPRESSION OF THE FIBRINOGEN-BINDING DOMAIN ON THE STAPHYLOCOCCUS AUREUS CELL SURFACE.

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Clumping factor of *Staphylococcus aureus* is a fibrinogen-binding protein which is located on the bacterial cell surface. The protein has an unusual repeat domain (region R) comprising mainly the dipeptide aspartate and serine. To determine if region R has a role in the surface display of the fibrinogen-binding region A domain, deletions lacking the region R encoding region of the *clfA* gene were generated. To determine the minimum length region R required for wild-type levels of ClfA expression, variants with truncated region R domains were constructed. *S. aureus* cells expressing mutated *clfA* genes were tested for (1) proteins releasable by lysostaphin treatment that reacted with antisera specific for region A, (2) clumping in soluble fibrinogen, (3) adherence to immobilised fibrinogen and (4) expression of the ClfA antigen on the cell surface by fluorescent activated cell sorting analysis. Each construct expressed three major immunoreactive protein, two of which were putative N-terminal degradation products. Greater than 40 region R residues were required between region A and W (72 residues between region A and the LPDTG sorting signal) for wild-type levels of clumping in fibrinogen. A stepwise decrease in clumping titre was observed as the distance between region A and LPDTG was decreased from 72 to 4 residues. Similarily, a decrease in binding of anti-ClfA serum and in binding to fibrinogen-coated plastic surfaces was observed with cells expressing ClfA with 40 region R residues or less. Nevertheless, low levels of adherence to fibrinogen and binding to anti-ClfA serum occurred with ClfA derivatives that lacked region R altogether. This indicates that a small proportion of the ClfA molecules are linked to peptidoglycan very close to the cell surface but that greater than 72 residues are needed to allow sufficient ClfA molecules to span the entire cell wall and to display the biologically active A domain in a form that can participate fully in fibrinogen binding.

# IDENTIFICATION OF PHOSPHATIDYLINOSITOL MANNOSIDE (PIM) AS A MYCOBACTERIAL ADHESIN MEDIATING BOTH DIRECT AND OPSONIC BINDING TO MAMMALIAN CELLS

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The molecular basis for the binding of *Mycobacterium tuberculosis* (*M.tb.*) to nonphagocytic cells is incompletely understood. We found that Chinese hamster ovary (CHO) cells and primary porcine aortic endothelial cells were able to bind *M.tb.* strain H37Rv efficiently in vitro, and that binding was markedly enhanced by 10-20% human or bovine serum. Preincubation with individual candidate opsonins revealed that recombinant human mannose-binding protein (rMBP), fibronectin, and transferrin were each able to enhance binding 3-fold. Preincubation of bacteria in serum depleted of mannan-binding lectins or in genetic MBP-deficient serum resulted in binding enhancements that were only ~60% of that produced by preincubation in control serum. In contrast, serum depleted of fibronectin or transferrin retained its opsonizing capacity, suggesting that the latter two are not significant opsonins in whole serum. Binding of *M.tb.* and *M. smegmatis* to both CHO and endothelial cells was blocked (60-70%) in the presence and absence of serum by a monoclonal antibody, MAb 1D1. The 1D1 antigen was purified from mycobacterial cell walls and chemically identified as phosphatidylinositol mannoside (PIM). Latex beads coated with purified 1D1 antigen bound to CHO cells, which was enhanced 3-fold by serum and abolished by periodate treatment, suggesting a requirement for the PIM mannoses in opsonic adhesion. This was likely mediated, at least in part, by serum MBP, as rMBP bound strongly to the 1D1 antigen in a TLC overlay assay. Thus, surface-exposed PIM may function as a mycobacterial adhesin.

# IDENTIFICATION OF ACID-INDUCIBLE GENE EXPRESSION IN HELICOBACTER PYLORI

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*Helicobacter pylori* is a gram negative, microaerophilic, bacterium that was first isolated in 1982 from the gastric mucosa of several patients suffering from gastritis. *H pylori* infection is associated with mild gastritis, duodenal ulcers, and gastric carcinomas. In order to elucidate the molecular mechanisms involved in the pathology of the organism, a better understanding of its basic biology in the *in vivo* environment is necessary. Since *H pylori* infects via an oral route subsequent to colonization of the gastric mucosa, a condition that is undoubtedly encountered by the organism is low pH.

We have developed a genetic selection/screen that utilizes inducible *H pylori* urease expression and activity to identify genes that are specifically regulated by exposure to low pH. Using DNA obtained from a clinical isolate, we have constructed an *H pylori* library that drives the expression of a promoterless *ure*B gene, one of the two structural subunits of *H pylori* urease. This library has been transformed into a urease (-) *ure*B deletion strain of *H pylori* . We have shown that this deletion strain can be complemented for urease activity if a wild type copy of *ure*B is expressed in trans. Urease catalyzes the reaction: urea---> ammonium + $CO_2$, which results in a net increase in pH. The selection takes advantage of the inability of urease (+) organisms to grow at a pH=7 in the presence of urea. Using this medium, we have shown that we are able to select against constitutive promoters that have been cloned upstream of the *ure*B gene. Colonies that arise on medium containing urea represent library members that either do not have a promoter cloned upstream of the *ure*B or contain promoters that are regulated. We will present data on acid regulated genes identified using the above approach.

# *MYCOBACTERIUM TUBERCULOSIS* ADENYLYL CYCLASE: A EUKARYOTIC-LIKE PROTEIN SIGNALING COMPONENT.

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Eukaryotic-like adenylyl cyclase has been identified in *Mycobacterium tuberculosis* $H_{37}R_{v}$. The *M. tuberculosis* adenylyl cyclase gene (*cya*) was cloned by complementation of a cAMP dependent expression of maltose operon in a *cya* defective *E. coli* strain. The nucleotide sequence analysis of the *cya* gene revealed an open reading frame that coded for 444 amino acids. A comparison of *M. Tuberculosis* adenylyl cyclase with the eukaryotic Type I and the calmodulin responsive adenylyl cyclases revealed an average of about 40% identical and 60% similar amino acids. These matches were extensive in the cytoplasmic domains of eukaryotic adenylyl cyclase. The occurrence of eukaryotic-like adenylyl cyclase in *M. tuberculosis* suggests a role for this important enzyme in cell signaling and perhaps in the pathogenesis of *M. tuberculosis*. We suggest that the human pathogen *M. tuberculosis* might have acquired the eukaryotic *cya* by a recombination event.

# DNA ANALYSES OF VLS (VMP-LIKE SEQUENCE) OF *BORRELIA BURGDORFERI* SENSU STRICTO AND ANTIBODY RESPONSE OF LYME DISEASE PATIENTS TO VLS.

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Lyme disease is a multiorganic infectious disease caused by the tick-borne spirochete *Borrelia burgdorferi* sensu lato, which is transmitted to human by *Ixodes* ticks. *B.hermsii* causes relapsing fever, changing their surface variable major protein (Vmp), and escaping from host-immunized system. The agents of relapsing fever and Lyme disease are spirochetes of the genus *Borrelia.* However, *vmp* homolog have not found in Lyme disease *Borrelia*, except for *ospC*, so *ospC* has been thought as a homolg of the *vmp* .

The DNA fragments containing the Vls (VMP-like sequence) of *B. burgdorferi* were cloned from strain 297 and were analyzed by Inverted-PCR, PFGE, Southern hybridization, Northern hybridization, and RT-PCR. These Vls fragment existed as direct-repeating units on borrelial plasmid, not chromosome and the other plasmids. One Vls-unit of strain 297 was about 669 bases in sequence, and predicted peptide length was 223 amino acids. Vls was similar to *vmp* of *B.hermsii*, than *ospC* of *B.burgdorferi.* Vls transcription was detected by RT-PCR but not by Northern hybridization, which suggested that Vls expression was at a quiet low level in culture condition. Forthermore, recombinant Vls was constracted in *Escherichia coli* JM109, and antibody against this recombinant Vls was detected in three of five patients in USA, by immunoblotting. These results suggested that Lyme disease *Borrelia* expressed the Vls, similar to Vmp that is involved in escaping from host immunosystem, in patients.

# AVRRPM1 FUNCTION IN PLANT AND BACTERIAL CELLS

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A primary model of plant resistance to a bacterial pathogen describes the presence of an avirulence (*avr*) gene from bacteria and a resistance (*R*) gene in plants. The presence of both these genes during a pathogenic attack culminates in a hypersensitive response (HR) characterized by collapsed and necrotized tissue. While a multitude of *avr* genes have been cloned, their primary amino acid sequences and tertiary structures do not reveal a possible mechanism of action of the protein (with the exception of *avrD*). We are using the genes *RPM1* and *avrRpm1*, contained within Arabidopsis (Col-0) and *Pseudomonas syringae* pv. maculicola (Psm), respectively, to understand the recognition events within the plant and the bacteria that lead to a host-specific HR. Native as well as c-myc-tagged *avrRpm1* constructs expressed in bacteria or *in planta* have shown us that the epitopes interfere with avrRpm1 delivery from bacteria. However, when *avrRpm1* transgenic plants are crossed into an RPM1 background, the resulting F1 plants display a dwarfed, sickly, phenotype, suggesting that even the epitope tagged *avrRpm1* is still functional within the plant cell. Immunoblots of c-myc-tagged avrRpm1 suggest that the protein may be post-translationally modified *in planta*, perhaps via a phosphorylation event. Further experiments characterizing the interaction of avrRpm1 with other plant proteins will be described.

*avrRpm1* has been shown to be a virulence factor in PsmM2, that is, its presence is required for growth on rpm1 null ecotypes. However, we don't know if it is a virulence factor in other strains of Psm, such as PsmM6. Interestingly, the bacteria that are derived from a PsmM6/Col-0 incompatible interaction are composed of two morphologies. The new morph has smooth-edged colonies and is now fully virulent on RPM1 plants. The other morph gives the parental rough-edged colonies and is intermediate in virulence. We have found that this phase change to virulence is completely dependent on RPM1 function of the plant since either an isogenic rpm1 mutant or an rpm1 null does not give rise the virulent morph. Additionally, avrRpm1 in PsmM6 is chromosomal, while the smooth morph carries its (non-functional) *avrRpm1* on a plasmid of ~35 kb. We believe that an integrative plasmid carrying *avrRpm1* is able to excise in response to an RPM1 dependent signal. Experiments mapping the excision event and characterization of the nature of avrRpm1 protein from PsmM6 will be described.

# GENETIC ANALYSIS OF VIRULENCE OF *PSEUDOMONAS SYRINGAE* ON *ARABIDOPSIS THALIANA*

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Plant-pathogen interactions are highly selective and involve a continuous exchange of signals. The specific functions responsible for microbial pathogenesis on plants, including those required for determining host range, initiation of infection, and survival *in planta* are not clearly defined. To address these questions, our lab employs the interaction between the crucifer *Arabidopis thaliana* and *Pseudomonas syringae* pv. *tomato*, the causitive agent of bacterial speck disease of tomato. Tn5-mutagenized lines of *P. syringae* pv. *tomato* have been screened for altered pathogenicity responses when inoculated on susceptible ecotypes of *A. thaliana* and tomato. Several pathogenicity mutants have been identified and categorized into classes. One class of mutants shows host specific virulence, exhibiting reduced pathogenicity on *A. thaliana* but strong symptom production on tomato. Another Tn5 insertion mutant showed a dramatic loss of virulence on both hosts and carries a disruption in a homolog of *DsbA* , a gene involved in periplasmic disulfide bond formation. Further characterization of these and other mutants should provide insight into the determinants of microbial pathogenicity on plant hosts.

# THE ROLE OF *PSEUDOMONAS AERUGINOSA* PHOSPHOLIPASE C AND LIPASE IN ACUTE AND CHRONIC INFECTION

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The sequences of pathophysiological events leading to chronic *Pseudomonas aeruginosa* infection of the lung in Cystic Fibrosis (CF) patients are not yet known. Colonization with *P. aeruginosa* is correlated with an early increase in serum antibodies against the two lipolytic *P. aeruginosa* enzymes, the phospholipase C and lipase. To analyze the molecular requirements leading to chronic *P. aeruginosa* infection we studied the interaction of *P. aeruginosa* strains isolated from CF patients at the onset, 2-3 years and 5 years after the onset of infection with human neutrophils and human monocytes. *P. aeruginosa* isolates from the acute but not from the chronic infection phase, also belonging to the same clone, express high phospholipase C (PLC) activities, are potent inducer for leukotriene $B_4$ generation, for enzyme release, but suppress proinflammatory cytokine release. Purified hemolytic, but not non-hemolytic, PLC from *P. aeruginosa* induces dose-dependently the release of the chemotactic lipid mediators LTB4 and 12-HETE, of proteolytic enzymes, and the vasodilatator histamine. Low concentrations of PLC (<1 unit/$10^6$ cells) induced proinflammatory cytokine release (IL-8, IL-6, TNF-alpha, IL-1ß) and IL-10 release. In contrast to PLC the lipase is synthesized during acute and chronic P. aeruginosa infection. Therefore we studied a) the effect of *P. aeruginosa* lipase as inflammatory mediator release from human peripheral blood mononuclear cells (PBMC) and b) the effects of human peripheral blood mononuclear cells on lipase expression and production in *P. aeruginosa*. In our experiments we used *P. aeruginosa* PAO1, as well as a lipase-negative and a lipase-overexpressing (20 fold) mutant of PAO1. Our data clearly show that lipase induces the release of arachidonic acid metabolites but suppresses proinflammatory cytokine release. In this regard, TNF secretion from human PBMC, measured by ELISA and PCR, was negatively correlated with lipase production in *P. aeruginosa*. Human ibflammatory cells (neutrophils, peripheral blood mononuclear cells did not significantly modulate lipase promotor activity, lipase production or lipase activity. Our results may contribute to the development of anti-pseudomonal strategies.

# ADHERENCE OF *PEPTOSTREPTOCOCCUS MICROS* TO ORAL EPITHELIAL CELLS

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*Peptostreptococcus micros*, a gram-positive anaerobic coccus, is associated with periodontitis and it is also isolated from non-oral mixed anaerobic infections. Two types of *P. micros* can be isolated from the periodontal pocket; the smooth (Sm) type and the rough (Rg) type. The Rg type is characterized by fibrillar surface structures, which are absent on the Sm type surface. The Rg type readily converts to a smooth-rough variant, which also lacks the surface structures. To study the involvement of the fibrillar surface structures of the Rg type, we characterized the adherence of both types and of smooth-rough variants to KB and HeLa epithelial cell lines and freshly isolated gingival epithelial cells by light-microscopy. In addition, the influence of enzyme treatment, pH and incubation with serum, saliva, sugars, or other possible inhibitors on the adherence was examined.

The Rg type had significantly lower adherence capacity compared to the Sm type or smooth-rough variants of *P. micros*. Multiplicities of infection of 10 resulted in adherence numbers of 4.6 $\pm$ 1.4 bacteria per cell for the Rg type compared to 7.1 $\pm$ 0.8 for the Sm type and 7.1 $\pm$1.7 for the smooth-rough variants. Adherence of *P. micros* to the 3 epithelial cell types was similar. Treatment of the bacteria with periodate resulted in a marked decrease in adhering bacteria, whereas protease treatment had no effect on the adherence of the 3 types. Changes in pH, as well as addition of sugars, gelatin, BSA or Tween20 mixtures did not influence the adherence of the 3 types of *P. micros*; preincubation of the bacteria with pre-immune sera or specific antisera did not have any effect on the adhesion of *P. micros* either.

In conclusion, adherence of *P. micros* to epithelial cells seems to be dependent on extracellular polysaccharides. Until now we have no evidence for a specific interaction between bacteria and the epithelial cell. The lower adherence capacity of the Rg type *P. micros* may be caused by the presence of the fibrillar structures, forming a spatial obstruction between bacteria and epithelial cells. The function of these fibrillar structures remains unclear.

# PHASE VARIATION IN A H.PYLORI PROTEIN BY FRAMESHIFT MUTATION

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*Helicobacter pylori* is a gram negative microaerophilic organism that colonizes the human stomach and plays an active role in chronic gastritis. During H.pylori infection of humans there is intimate binding of the organism to the apical plasma membrane of surface epithelial cells in regions where the microvillus membrane is disrupted. Several putative adhesins have been identified which may contribute to its adherence to gastric mucosa. In this communication we demonstrate the characterization of a possible operon that codes for several proteins that might play a role in adhesion/antigenicity. We show the presence of a unique sequence in a gene which consists of a stretch of G residues positioned in the region encoding the signal peptide that is similar to the sequence seen in *N. gonorrohoeae* pilC gene which codes for a N. gonorrohoeae adhesion protein(EMBO Journal .10:477-488). This unique sequence seems to control the expression of the corresponding protein at the translational level by frameshift mutations. This operon/gene is highly conserved across *Helicobacter* species ( from *Helicobacter pylori* to *Helicobacter mustelae*) as seen by genomic sequencing. One of the open reading frame codes for a 30kD protein which is preceded by a typical Shine-Dalgarno sequence. The sequence following the AUG codon would encode a typical membrane spanning hydrophobic N terminal region followed by a long hydrophilic region. A tract of 12 G residues was found in the region encoding the putative signal peptide for this protein analogous to the situation seen in *Nessieria* pilC gene. Deletion of one G residue would align the long open reading frame with the AUG codon in frame 2. The translated region in frame 1 and 3 would terminate the protein prematurely. The data therefore suggest that the expression of this probable surface antigen is regulated by frameshifting in the region encoding the signal peptide. All three possible reading frames are present in the population as detected by variation in the length of the PCR product caused by slippage in the 12G residues encoding the putative signal peptide.

## *H. PYLORI* CONTAINING CYTOPLASMIC UREASE ONLY ARE ACID SENSITIVE

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*Helicobacter pylori* colonizes the human stomach and plays a decisive role in peptic ulcer disease. We have recently demonstrated that *H. pylori* urease, an essential colonization factor, is simultaneously present within *both* the cytoplasm and on the surface of the bacterium *in vitro* and *in vivo*. We have shown that surface localization of *H. pylori* urease is dependent upon bacterial autolysis. In this report, we demonstrate that *H. pylori*, carrying active cytoplasmic urease but little or no surface urease are acid sensitive. To prove this we used flurofamide, a poorly diffusable inhibitor of urease activity, to preferentially inactivate extracellular and surface associated urease. *H. pylori* treated with flurofamide for 10 min. were no longer acid resistant in the presence of 5mM urea, indicating that surface associated and extracellular urease play a key role in acid resistance. In a separate experiment, an early log phase culture of *H. pylori* with little surface associated (and extracellular) urease as detected by immunolocalization experiments, was acid sensitive even in presence of 5mM urea unlike late log phase culture, by four orders of magnitude. Further, *E. coli* carrying *H. pylori* urease genes which produces enzymatically active urease located in the cytoplasmic compartment, are also acid sensitive. Thus, we conclude that *H. pylori* urease present on the surface and in the extracellular compartment is required for neutralization of the low pH levels in the gastric mucosa. Because surface associated and extracellular urease are released due to bacterial autolysis and not exported by an active export mechanism (*Infect. and Immun.* 64:905-912 and 65:1181-1188), our results show the pivotal role that bacterial autolysis plays in pathogenesis of *H. pylori*.

## THE *PRY* GENES: HOMOLOGS OF PLANT PATHOGEN-RELATED PROTEINS

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The *PRY1* gene was identified as part of a random clone sequencing project of a yeast meiosis-specific cDNA library and shares strong sequence similarity with two open reading frames, which we call *PRY2* and *PRY3*, that were identified as part of the yeast genome sequencing project. These proteins share roughly 60% identity, and over 80% similarity, in a 140-residue region of these proteins. These proteins also share significant regions of homology with a class of plant pathogenesis related proteins, called PR-1. PR proteins are host-encoded polypeptides which are strongly expressed in response to a pathological or related stress conditions. Genes that share sequence homology with the plant and yeast proteins have also been found to be expressed in mammalian testes and in human astrocytic brain tumors. Given that these proteins are highly conserved among the different species ranging from yeast to plants and humans, it seems plausible that they are involved in evolutionary conserved processes. The plant proteins are secreted and the yeast proteins have N-terminal signal sequences and potential O-glycosylation sites, which suggests that they may also be secreted. In addition, one of the proteins contains a GPI anchor sequence, suggesting that it may be attached to the membrane or is a component of the cell wall. Data will be presented on the cellular localization of these proteins.

The *PRY1* and *PRY2* are strongly induced under starvation conditions. However a triple *pry1 pry2 pry3* null mutant does not appear have any effect on viability or growth under normal, starvation or other stress conditions. The triple null mutant has no apparent abnormalities in the cell wall or morphology. We are currently searching for mutants, which in combination with the *pry* null mutations, cause a lethal phenotype.

VIRULENCE AND HYPHAL FORMATION OF *CANDIDA ALBICANS* REQUIRE THE Ste20p-LIKE PROTEIN KINASE CaCla4p

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The *CaCLA4* gene of *Candida albicans* was cloned by functional complementation of the growth defect of *Saccharomyces cerevisiae* cells deleted for both the *STE20* and *CLA4* genes. *CaCLA4* encodes a member of the Ste20p family of serine/threonine protein kinases and is characterized by a pleckstrin homology domain and Cdc42p binding domain in the amino-terminal non-catalytic region. Deletion of both alleles of *CaCLA4* in *C. albicans* caused defects in hyphal formation *in vitro* in synthetic liquid and solid media, and *in vivo* in a mouse model for systemic candidiasis. The deletions reduced colonization of kidneys in infected mice and completely suppressed *C. albicans* virulence in the mouse model. This phenotype is different to that previously observed for deletion of the *CST20* alleles encoding the other known member of the Ste20p family of protein kinases in *C. albicans* (Leberer et al., PNAS 93, 13217-13222, 1996).

Our results suggest that hyphal formation of *C. albicans* mediated by the CaCla4p protein kinase may contribute to the pathogenicity of this dimorphic fungus. The formation of hyphae could be part of a survival strategy of *C. albicans* cells to escape attacks of the cell-mediated immune system in the host and/or facilitate invasion of *C. albicans* cells into infected tissues. Thus, CaCla4p and additional regulators of hyphal formation may be valid targets for the development of new therapeutic antifungal strategies.

# *E. FAECALIS* ALKALINE PHOSPHATASE AS A REPORTER OF SUBCELLULAR LOCATION

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We are interested in identifying virulence factors in Group B Streptococcus (GBS), the most common pathogen causing neonatal bacteremia or meningitis. Reasoning that host/pathogen interactions are likely to involve secreted proteins or membrane proteins, we have been testing a screening method to identify these proteins in GBS. The two "reporter enzymes" most commonly used for this purpose, *E. coli* alkaline phosphatase and beta lactamase, could not be used. The *E. coli* alkaline phosphatase loses much of its activity when expressed in Gram positive organisms (1), perhaps due to the lack of disulfide bond forming activity in the extracytoplasmic space. The beta lactamase protein can not be used, since this antibiotic is still an important defense against GBS infection. In contrast, we found that the *Enterococcus faecalis* alkaline phosphatase (AP) confers a blue phenotype on GBS colonies grown on plates containing a chromogenic substrate, 5-bromo-2-chloro-3-indolylphosphate (XP) (2). Wild type GBS has little endogenous phosphatase activity, and forms white colonies on XP plates. The quantitative assay for alkaline phosphatase activity has been adapted for GBS. A GBS strain expressing AP from a high-copy number plasmid has an activity roughly 1000-fold higher than an isogenic strain without AP. When the signal sequence of AP is deleted the activity is reduced 16 fold. We have interpreted this result to indicate that the activity of AP is sensitive to cytoplasmic location. Lysis of the bacteria, which is needed when Gram negative bacteria are being assayed, is not needed in GBS. Experiments are underway to test AP activity when fused to heterologous signal sequences or to transmembrane domains of integral membrane proteins. We expect that this reporter enzyme will provide a genetic means of identifying exported proteins in GBS.

# ACTIVATION OF NF-KAPPA B VIA A SRC-DEPENDENT MAPK-RSK PATHWAY BY P. AERUGINOSA IS REQUIRED FOR UPREGULATION OF MUCIN TRANSCRIPTION IN THE PATHOGENESIS OF CYSTIC FIBROSIS

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Pseudomonas aeruginosa is a major opportunistic pathogen in cystic fibrosis lung diseases. It causes chronic lung infection and mucus overproduction which characterize CF lung disease (J.D. Li, et el., Proc. Natl. Acad. Sci. U.S.A. 94:967-972, 1997). The molecular mechanisms underlying interactions between P. aeruginosa and surface epithelial cells, and how the bacterial virulence factors activate host epithelial cell signal transduction pathways and cause the clinical problems are unclear. Here we show that P. aeruginosa activates a src-dependent Ras-Raf-MAPK pathway, which, in turn activates NF-kappa B via activation of pp90RSK. The activated NF-kappa B binds to kappa B site in the promoter region of human mucin MUC2 gene and activates mucin transcription. These studies may provide new insights into understanding mechanisms underlying molecular interactions between bacterial virulence factors and host epithelial cell signal transduction pathways and may lead to the new therapeutic strategies for cystic fibrosis.

# ROLE OF THE HYPERVARIABLE REGION IN STREPTOCOCCAL M PROTEINS

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All strains of *Streptococcus pyogenes* (group A streptococcus) share the ability to grow rapidly in human blood, a property that is due to the expression of surface M proteins with antiphagocytic function. A characteristic feature of M proteins is the presence of a "hypervariable" N-terminal region, which shows very extensive sequence variation between M proteins expressed by different strains. The hypervariable region is a major target for the immune system, but the function of this important region has remained unknown. We have studied four different M proteins and found that their hypervariable regions bind a specific ligand, the human complement inhibitor C4BP. This high-molecular-weight plasma protein belongs to a group of structurally related proteins that regulate the human complement system. The ability of the hypervariable region of several M proteins to bind C4BP may explain why these M proteins have antiphagocytic function, since the bound C4BP may prevent opsonization and thereby prevent phagocytosis.

Our working hypothesis is that the binding of C4BP is of major importance for the resistance to phagocytosis. The hypervariability of the N-terminal region of M-proteins can then be seen as the result of two opposing forces: one favoring sequence changes and antigenic variation, the other limiting variation due to the requirement to retain a tertiary structure permitting binding of the ligand.

Although many strains of *S. pyogenes* express M proteins that bind C4BP, other strains lack this property. However, several such strains have been reported to bind factor H, another complement regulator present in plasma. Taken together, these data suggest that *S. pyogenes* can use either C4BP or factor H to achieve the same result, i.e. resistance to phagocytosis. However, factor H has been reported to bind outside of the hypervariable region. Possible explanations for this situation will be discussed.

## A TWO-COMPONENT REGULATORY SYSTEM REQUIRED FOR *BRUCELLA ABORTUS* VIRULENCE

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*Brucella* spp. are facultative intracellular pathogens of both humans and animals. The virulence of some gram-negative bacteria is linked in part to resistance to bactericidal cationic peptides, and it has been proposed that the unusually high resistance of *Brucella* to these peptides contributes to its ability to survive within phagocytes. In an attempt to identify *B. abortus* genes involved in such a resistance (and possibly in virulence), Tn5 was used to generate insertion mutants that were screened for increased sensitivity to polymyxin B. Two polymyxin B-sensitive mutants that retained a smooth-type lipopolysaccharide were counterselected. These mutants were also remarkably sensitive to other cationic peptides tested (melittin and poly-L-lysine), and simultaneously showed increased outer membrane permeability to detergents and hydrophobic probes. As compared to both the parental strain (fully virulent) and *B. abortus* B19 (attenuated vaccine strain), both mutants were avirulent in a mouse model.

DNA sequence analysis of the region affected by the mutation revealed two transcriptional units, which were called *bvr*S and *bvr*R (brucella virulence related), and which encoded the members of a two-component sensory transduction system. The membrane-spanning sensor (BvrS) was a histidine protein kinase, and the response regulator (BvrR) showed similarity to the OmpR family of regulators. The *bvr*S-*bvr*R system showed high homology with the *chv*I-*chv*G two-component regulatory system described for *Agrobacterium tumefaciens* (a plant pathogen) and *Rhizobium meliloti* (a plant facultative endosymbiont), two bacteria phylogenetically related to *B. abortus*. Significantly, insertion mutans in the *chv*I-*chv*G system of *A. tumefaciens* have also been shown to be avirulent. The strong identity (>84%) of the sequences, the relevance of the genes in *B. abortus* and *A. tumefaciens* virulence, and the association of the above bacteria with both animal and plant eukaryotic cells, suggest that those genes play a key role with respect to the intracellular behavior of this prokaryotic group.

# IDENTIFICATION OF NOVEL STAPHYLOCOCCAL VIRULENCE GENES BY *IN VIVO* EXPRESSION TECHNOLOGY.

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Morbidity and mortality from staphylococcal infections are high, and emergence of resistance to currently available antibiotics poses a major threat to public health. There is an immediate need for alternate approaches to prevention and therapy of *S. aureus* infections.

*In vivo* expression technology (IVET) is a strategy to identify bacterial genes that are up-regulated during mammalian infection. Rather than relying on laboratory media to simulate the environment of infected mammalian tissue, IVET uses infected tissue itself as a selective medium. Some *ivi* (in vivo induced) genes presumably encode virulence factors crucial to infection, factors which might be useful as screening targets for antibiotic development or as immunogens in novel vaccines.

A reporter cassette was developed based on *tnpR*, a gene encoding the enzyme resolvase. This enzyme catalyzes excision of an antibiotic resistance marker flanked by direct repeats of *res*, the sites at which resolvase acts. The reporter was integrated in single copy into the *S. aureus* chromosome. Plasmid pESAL2 is a shuttle vector that permits formation of transcriptional fusions between cloned DNA and *tnpR*. A genomic staphylococcal library was created in pESAL2 and transduced into the reporter strain, where transcriptionally active fusions direct resolvase-mediated excision of the antibiotic marker. The loss of antibiotic resistance in descendant progeny serves as a permanent hereditable marker of prior gene expression.

Using this system in a murine renal abscess model, we identified 69 staphylococcal genes that are induced during infection. Of them, only 8 represent known staphylococcal genes; 16 others have homology to known non-staphylococcal genes. The known staphylococcal genes include *agrA*, a key locus regulating numerous virulence products, and *lip* (a glycerol ester hydrolase), which may enhance staphylococcal survival in abscesses. We constructed eleven strains containing mutations in previously unknown *ivi* genes. Of those strains, seven were significantly attenuated in virulence compared to the wild type parent. The mutagenized *ivi* genes may encode novel staphylococcal virulence factors. When we attempted to create interruptions in 4 other genes, no mutants could be isolated, suggesting that these genes perform an essential function in the cell. These genes are currently being investigated further.

# USING *PSEUDOMONAS AERUGINOSA* AND A *CAENORHABDITIS ELEGANS* MODEL TO DEFINE MOLECULAR INTERACTIONS REQUIRED FOR BACTERIAL PATHOGENICITY

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We are studying the interactions between *C. elegans* and *Pseudomonas aeruginosa*, an opportunistic human pathogen. We have found that a clinical isolate of *P. aeruginosa*, strain UCBBPP-PA14, can kill *C. elegans* by at least two distinct mechanisms. In minimal medium, PA14 infects worms and kills them over a period of 2-3 days ("slow killing). In contrast, when worms are exposed to PA14 grown in rich medium they become sluggish, paralyze and die within 4-24 hours ("fast killing"). Evidence that slow and fast killing are mechanistically distinct comes from the characterization of bacterial mutants that have dramatically different effects in the two conditions.

Two lines of evidence suggest that fast killing is mediated by diffusible toxins. First, live bacteria are not required to mediate this effect. Second, mutations in *C. elegans* genes encoding P-glycoproteins, which result in increased sensitivity to toxins such as colchicine, also render the worms more sensitive to fast killing by PA14. In contrast, slow killing is mediated by live bacteria only and P-glycoprotein mutations have no effect under these conditions. These results suggest that slow killing might involve an infectious process, a hypothesis we are exploring by following the fate of PA14 expressing the green fluorescent protein (PA14-GFP) *in vivo*. Preliminary results indicate that PA14-GFP bacteria accumulate to high levels in the lumen of the *C. elegans* digestive tract. This is not seen with control GFP-expressing *E. coli*. In addition, a GFP-expressing mutant containing an insertion in the PA14 *gacA* gene, a known transcriptional regulator of virulence factors that shows extremely attenuated levels of slow killing, also shows no accumulation in the gut.

We screened for bacterial mutants defective in fast killing to identify the toxins that mediate this killing. Three mutants from this screen are defective in the production of phenazines, identifying these as one class of toxin. We have also identified mutants that produce normal levels of phenazines and are currently characterizing them. All these mutants are currently being tested in both the plant and the mouse models and preliminary results show that the *C. elegans* fast killing model can be effectively used to identify virulence factors that are relevant to pathogenesis in both plant and mammalian hosts.

In order to gain insight into the response of *C. elegans* to bacterial toxins, we have undertaken a mutagenesis screen to identify mutations that confer resistance to fast killing and so far have identified 6 resistant mutants. This analysis should provide useful information about the interactions between bacterial factors and host targets that determine the outcome of bacterial-host confrontations.

## T HELPER CELL RESPONSES IN OPPORTUNISTIC FUNGAL INFECTIONS: IMPLICATIONS FOR IMMUNITY AND THERAPY

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Patterns of susceptibility and resistance to opportunistic fungal infections have been associated with differential activation of Th1 and Th2 cell responses, respectively (1). Activation of an appropriate Th cell response may also allow fungal pathogens to evade host antifungal resistance, which may result in fungal persistence and fungal-related pathology (2). In experimental models of *Candida albicans* and *Aspergillus fumigatus* infections, development of Th1 cell responses leads to resistance and onset of protective immunity, whereas activation of Th2 cell responses is associated with downregulation of protective Th1-dependent immunity and susceptibility to infection and pathology. Induction of anticandidal Th1 responses requires i) blockade of the CD28-B7 costimulatory pathway; ii) blockade of IL-4- and IL-10-dependent Th2 cell activation and iii) the coordinate action of selected cytokines, including IL-12. The balance of IL-4 and IL-12 may influence Th polarization as well as Th stability. Thus, early inhibition of IL-4, mainly produced by $CD4^+$ TCR $V\beta8^+$ T cells, results in the onset of IL-12-dependent protective Th1 responses. Early impairment of IL-12 production occurs in mice depleted of IL-12-producing neutrophils and prevents the occurrence of Th1 responses which are restored by replacement therapy with IL-12. However, IL-4 appears to be required for maintenance of memory anticandidal Th1 responses, through sustained IL-12 production. These data indicate that a thorough appreciation of the Th cell dichotomy in immune responses to fungi may have important implications for Th-directed immunotherapeutic strategies.

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# STAPHYLOCOCCAL ENTEROTOXIN B (SEB) PRIMES $CD8^+$ INTERFERON-GAMMA (IFN-γ) SECRETION IN RESPONSE TO NATIVE BACTERIAL ANTIGENS

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Superantigens have been recognized as important factors in bacterial pathogenicity. Superantigens, e.g., SEB, bind specific variable beta (Vβ) regions of the T cell receptor heterodimer distinct from the site of conventional antigen binding. This interaction stimulates cellular proliferation and cytokine secretion by the T cells.

We have developed a novel splenocyte-bacteria co-culture assay which allows the study of host-pathogen interactions in the context of the interdependent multicellular immune response. With this assay system, we can assess how superantigen exposure alters the host response to native, conventional bacterial antigens that might be encountered in the body.

Balb/c mice were injected with 50μg SEB or buffered saline 2 days prior to *in vitro* splenocyte isolation and co-culturing with *Streptococcus mutans*. Splenocytes from SEB-pretreated mice produced 15-25 times more IFN-γ than cells from control animals, as measured by ELISA. Intracellular cytokine labeling of SEB-pretreated splenocytes from 20 hour co-cultures identified 8.4% $CD8^+$ and 2.3% $CD4^+$ T lymphocytes as dual-labeled for IFN-γ. $CD8^+$ depletion prior to splenocyte-bacteria co-culturing significantly decreased IFN-γ levels, confirming $CD8^+$ cell IFN-γ secretion in response to bacteria following SEB exposure. Labeling of the $V\beta8^+$ subpopulations revealed a greater depletion of $CD8^+V\beta8^+$ cells, two times higher than that of $CD4^+V\beta8^+$. These data combined support by-stander cell (non-Vβ8) activation and/or depletion following SEB treatment. SEB pretreated splenocytes were cultured with titrated amounts of *S. mutans* and supernatants were analyzed for IFN-γ production. Addition of $10^4$ viable bacteria to splenocyte cultures were needed for significant IFN-γ production, suggesting that the response is dependent on antigen load. IL-12 levels peak prior to IFN-γ secretion in SEB-pretreated splenocyte co-cultures. A 10 hour pre-incubation of control splenocytes prior to bacterial cell addition increases IFN-γ secretion. These data suggest that SEB primes a response to native bacterial antigens. The mechanism of this response involves enhanced activity of antigen presenting cells and reactive T cells, resulting in stimulation and cytokine release, which contributes to pathogenesis.

DNA IMMUNIZATION WITH *pspA* CAN ELICIT PROTECTION AGAINST PNEUMOCOCCAL INFECTION

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PspA is a protection-eliciting protein of *Streptococcus pneumoniae* and is required for full virulence of the pneumococcus. A plasmid, pKSD2601, was constructed in which *pspA*/Rx1 was cloned inframe with a fragment of the gene encoding RSVG. The *pspA*::*rsv*G fusion was under the control of the early CMV promoter. This construct should have directed the expressed PspA to the cell surface. Cytoplasmic expression of PspA was observed, but we failed to obtain surface expression as detected by immunofluorescent staining with anti-PspA antibodies. However, intramuscular immunization of BALB/c mice with pKSD2601 significantly protected the mice from lethal challenge with *S. pneumoniae* A66, a capsular type 3 pneumococcus. The immunized mice had a mean log of colony forming units/ml of 2.97±0.25 pneumococci circulating in their blood at 24 hours post intravenous challenge as compared to control mice which had a mean log colony forming units/ml of 4.95±0.59 pneumococci. Those mice with lower numbers of pneumococci subsequently survived the challenge. Only 30% of the immunized mice had detectable levels of PspA specific antibodies in their serum. No PspA specific antibodies were detected in the control mice which received injections of the plasmid vector with no pneumococcal insert. The pneumococcus is an important mucosal pathogen against which an effective vaccine is needed. DNA immunization offers the possibility of an inexpensive multicomponent vaccine.

THE ROLE OF *MGA* IN GROWTH PHASE REGULATION OF VIRULENCE GENES OF THE GROUP A STREPTOCOCCUS

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The group A streptococcus (GAS, *Streptococcus pyogenes*) is an important human pathogen that causes a variety of infections at different locations within the host. The multiple gene regulator of GAS, Mga, activates expression of several GAS virulence genes in an environmentally-controlled regulon that includes the genes encoding M protein (*emm*) and C5a peptidase (*scpA*) To determine whether growth phase affects the expression of *mga* (which encodes Mga) and other virulence-associated genes in GAS, total RNA was isolated from the serotype M6 GAS strain JRS4 at different phases of growth and transcript levels were quantitated by hybridization using radiolabeled DNA probes. Expression of *mga* and the Mga-regulated genes *emm* and *scpA*, was found to be maximal in exponential phase and shut off as the bacteria entered stationary phase, while the housekeeping genes *recA* and *rpsL* showed constant transcript levels over the same period of growth. Expression of *mga* from a foreign phage promoter in a *mga*-deleted GAS strain (JRS519) altered the wild-type growth phase-dependent transcription profile seen for *emm* and *scpA*, as well as for *mga*. Therefore, the temporal control of *mga* expression requires its upstream promoter region and the subsequent growth phase regulation of *emm* and *scpA* is Mga-dependent. A number of putative virulence genes in JRS4 were shown not to require Mga for their expression, although several exhibited growth phase-dependent regulation that was similar to *mga: slo* (which encodes streptolysin O) and *plr* (encoding the plasmin receptor/glyceraldehyde-3-phosphate dehydrogenase ). Still others showed a markedly different pattern of expression (the genes for the superantigen toxins MF and *speC*). These results suggest the existence of complex levels of global regulation sensitive to growth phase that directly control the expression of virulence genes and *mga* in GAS.

# ADHERENCE OF CANDIDA ALBICANS CELLS TO RESILIENT DENTURE LINERS: INHIBITION BY SALIVA AND LINER-INDUCED CHANGES IN MORPHOLOGY

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Denture stomatitis, an inflammation of the soft tissue underlying a denture, afflicts up to 67% of denture wearers and is frequently caused by infection. Candida albicans is the most common opportunistic fungal pathogen in the oral cavity and is consequently a common cause of stomatitis. Resilient liners, both denture liners intended for long-term use and tissue conditioners intended for short-term use, are frequently used to treat this condition. It is important that the liners do not provide a surface for attachment and colonization by microorganisms normally present in the oral cavity. We have developed an ELISA-based assay for measuring the adherence of C. albicans to liners. With this assay, we have compared the adherence of C. albicans cells to a number of resilient liners including both denture liners and tissue conditioners, prescription products and over-the-counter products, and silicon and acrylic materials. We have found that cells adhere to all of these materials and that this adherence can be drastically reduced by pretreating the liners with saliva. Preliminary work has identified acidic proline-rich protein 1 (PRP1) as the salivary protein most likely to inhibit adherence. Furthermore, culturing C. albicans cells in the presence of some liner products causes a change in cellular morphology. C. albicans is a dimorphic yeast which can exist as round yeast cells or as elongated hyphae. Environmental changes such as a change in temperature or pH can result in dimorphic transition. Both morphological forms are present in an active infection with an increase in the proportion of hyphal cells; consequently, the filamentous hyphal morphology is most closely associated with an increase in virulence. Our studies show that some liners prevent the formation of hyphal cells under normally inducing conditions. The most likely agent present in the liners that may be responsible for this effect is the antifungal agent undecylenic acid. We present data that suggests a possible mechanism for the change in morphology which may have significant clinical relevance.

SPORULATION-DEPENDENT EXPRESSION OF ENTEROTOXIN IN *CLOSTRIDIUM PERFRINGENS.*

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*Clostridium perfringens* causes large scale outbreaks of food poisoning, due to the production of a potent enterotoxin by sporulating cells in the intestinal tract following ingestion of contaminated foods. We have shown that the enterotoxin gene, *cpe*, is controlled at the transcriptional level and is highly induced during sporulation. A *C. perfringens-E. coli* shuttle vector carrying a fusion of a ~500 bp *cpe* promoter region fragment to the reporter gene *gusA* from *E. coli* also was induced during sporulation. Primer extension experiments using RNA from sporulating cells identified four potential 5' ends upstream of the *cpe* start codon (designated P1 to P4). Internal deletions in the cpe promoter region were made to isolate the promoters, except P1 and P2, which overlapped. P3 alone and P1 and P2 together showed significant levels of sporulation-dependent expression, but P4 alone showed almost none. A series of nested deletions of the *cpe* promoter were made. Deletions including P4 and P3 had little or no effect on the level of expression, while deletions to P1 and P2 drastically reduced the induction. In vitro transcription assays using whole-cell extracts from sporulating cells also showed strong evidence of transcription from P1-P3. Regions with homology to sigma E-dependent promoters from *Bacillus* upstream of the P3 and P2 5' ends and another with homology to sigma K-dependent promoters upstream of the P1 5' end were detected. Also, genes coding for *C. perfringens* homologs of Spo0A, SigE, SigG, and SigK have been cloned and are currently being used to construct mutations in the chromosomal copies by homologous recombination to test their effects on sporulation and *cpe* regulation.

# THE SPECIFICITY OF PROTEOGLYCAN RECOGNITION VARIES AMONG LYME DISEASE SPIROCHETES

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Lyme disease, a chronic multisystemic disorder that can affect skin, heart, joints and nervous system, is caused by *Borrelia burgdorferi* (sensu stricto), *B. garinii* and *B. afzelii*. Attachment of these spirochetes to various host tissues is likely to be a critical step in the colonization of multiple tissues, and previous work showed that proteoglycans and integrins mediate binding of *B. burgdorferi* to mammalian cells. In the current study, the structural requirements for proteoglycan recognition by Lyme disease spirochetes was investigated by inhibiting bacterial attachment with defined preparations of proteoglycan, and by assaying bacterial attachment to host cells after enzymatic removal of specific classes of proteoglycans from the host cell surface. The infectious *B. burgdorferi* (sensu stricto) strain N40 recognized heparin, and to a lesser degree, dermatan sulfate and heparan sulfate, but not chondroitin-4-sulfate or chondroitin-6-sulfate. Preparations of heparin of defined size were tested for inhibition of bacterial binding, and the length the heparin chain directly correlated with its potency as an inhibitor. The minimum chain length for heparin recognition by *B. burgdorferi* was 16 residues. Thus, the length of the glycosaminoglycan chain is a critical determinant of bacterial recognition.

Proteoglycan recognition by four other Lyme disease spirochetes, including representatives of *B. afzelii* and *B. garinii*, was also examined. Host cell binding by all of the strains could be inhibited by exogenous proteoglycan, or by the enzymatic removal of host cell glycosaminoglycan. *B. garinii* strain PBi and *B. burgdorferi* CA20-2A exhibited a similar profile of proteoglycan binding as strain N40, recognizing heparin, heparan sulfate, and dermatan sulfate, but not chondroitin-4-sulfate or chondroitin-6-sulfate. *B. afzelii* VS461 recognized the same three proteoglycans, but differed from the above strains by recognizing dermatan sulfate significantly better than heparan sulfate. *B. burgdorferi* HB19 (clone 1), a high passage noninfectious strain, recognized dermatan sulfate almost exclusively. Therefore, proteoglycans mediate the attachment of diverse Lyme disease spirochetes to mammalian cells, but the specificity of proteoglycan recognition varies among different strains.

GROWTH PHASE DEPENDENT REGULATION OF MYCOBACTERIAL INTEGRATION HOST FACTOR,

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Given the important roles of integration host factor (IHF) in DNA replication and regulation of gene expression in *Eschericia coli*, we undertook to identify and characterize its functional homolog in the mycobacteria. Mycobacterial integration host factor, mIHF, is the mycobacterial protein required by mycobacteriophage L5 for integration of the phage genome into the bacterial chromosome. An *in vitro* recombination assay was utilized to purify mIHF to homogeneity. Unlike other IHF proteins, mIHF is comprised of a single polypeptide sequence, and the single copy gene encoding this polypeptide was cloned and sequenced. Database comparisons show that the mIHF proteins of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* are highly conserved. The protein was overexpressed in *E. coli* and purified in large quantity. Also unlike previously characterized integration host factors, biochemical analysis via gel shift and DNAse I footprinting have demonstrated that mIHF binds to DNA in the absence of integrase protein without apparent sequence specificity. Like *E. coli* IHF, mIHF was shown to bind to bent DNA sequences. Also like IHF, gel filtration and chemical crosslinking experiments indicate that mIHF can form dimers and higher multimers in solution. Previous results have also indicated that mIHF is upregulated in logarithmically growing *M. smegmatis* . The results of genetic analysis to determine whether *mIHF* is an essential gene in *M. smegmatis* and the regulation of its intracellular concentration by the growth phase of Bacille Calmette-Guérin will be presented.

# PCR FINGERPRINTING FOR *CRYPTOCOCCUS NEOFORMANS* DIAGNOSIS

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*Cryptococcus neoformans* is an opportunistic yeast affecting immunocompetent patients. Cryptococcal meningitis is mortal for AIDS patients. New technology must be applied to detect early the yeast and provide the better treatment. DNA fingerprinting and polymerase chain reaction (PCR) with specific primer for hypervariable repetitive DNA sequences were used to analyze environmental isolates and clinical specimens. Using $(GTG)_5$ as primer we obtain DNA polymorphisms which allowed us discriminate 9 operational taxonomic units (OTU) or common genetics profiles among 26 environmental strains of *Cryptococcus neoformans* var. *neoformans*. These OTU were diverse among them and consistently repeated. It suggests that *Cryptococcus neoformans* var. *neoformans* have a high genetic divergence in the studied area and this method recognizes more specific difference among the strains. The same method was applied to fecal samples (24) of AIDS patients negative for *Cryptococcus neoformans* cultures on Niger Seed Agar. DNA profiles were not corresponding to *Cryptococcus neoformans*. Fecal samples were inoculated with dilutions of *Cryptococcus neoformans* ($10^0$-$10^8$ cells/ml) and demonstrate similar profiles on DNA fingerprinting. All experiments were conducted under the same amplification and DNA extraction conditions and produced similar profiles. DNA profiles were diverse among strains and highly conserved among repetitions. DNA-PCR fingerprinting method seems to be more sensitive recognizing differences among strains of *Cryptococcus neoformans*. Therefore, PCR fingerprinting might become a useful technique for *Cryptococcus neoformans* diagnosis.

# HUMAN NATURAL KILLER ANTIBODIES

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Human natural candidacidal antibodies (KAb) representing the internal image of a wide spectrum yeast killer toxin (YKT) have been detected in the vaginal fluid of women affected by candidiasis owing to immunization with specific YKT cell wall receptors (YKTR) of the infecting microorganism. KAb proved to be functionally equivalent in their biological activity to immunoprotective YKT-like antiidiotypic antibodies (KTIdAb) raised in the serum and vaginal fluid of animals by parenteral or mucosal idiotypic vaccination with the YKTR-like idiotype of a YKT-neutralizing monoclonal antibody (mAb KT4).

KAb and KTIdAb showed to compete with YKTR of *Candida albicans* cells and to transfer passive immunity to naive animals in a rat vaginitis experimental model and their properties were neutralized by mAb KT4.

KAb exerted a microbicidal activity against *Pneumocystis carinii* and a multidrug resistant strain of *Mycobacterium tuberculosis* by interaction with a putative YKTR transphyletic receptor. YKT-like antiidiotypic monoclonal (mAb-KTIdAb) and recombinant antibodies in the single chain format (ScFv-KTIdAb) produced from the spleen lymphocytes of mice vaccinated by mAb KT4 confirmed their candidacidal, pneumocysticidal and mycobactericidal activity *in vitro* and showed a therapeutic potential in animal models of experimental candidiasis and pneumocystosis.

MAb-KTIdAb and ScFv-KTIdAb competed with YKT and KAb for YKTR of YKT-sensitive microorganisms and their activity was neutralized by mAb KT4.

This is the first demonstration that natural antibodies with killer activity may be part of the human immune repertoire against infecting microorganisms.

# FLUORESCENCE-BASED ASSAY FOR MYCOBACTERIAL GENE EXPRESSION

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Although *Mycobacterium tuberculosis* was one of the first identified bacterial pathogens, relatively little is known about this bacterium and how it is able to cause disease. We developed a fluorescence-based assay using *lacZ* as a reporter gene to identify possible virulence genes and to study gene regulation in mycobacteria. We used multicopy and integrated derivatives of the *lacZ* fusion plasmid pJEM15 with the *gyrB* promoters from *M. smegmatis* and *M. tuberculosis*, and the *M. bovis* BCG hsp60 promoter to optimize the system. Several lipophilic, fluorescent ß-galactosidase substrates, including derivatives of fluorescein di-ß-d-galactopyranoside (FDG) with carbon chain lengths varying from 2-12, were compared for their abilities to detect specific gene expression in *M. smegmatis* and BCG bacteria. An important advantage of this approach is that these substrates penetrate live cells eliminating the need for cell lysis. This feature reduces manipulations and enables time course experiments to be performed. Substrates were added directly to culture medium and fluorescence was measured using a Cytofluor 2350 fluorescence plate reader. Overall, we found that signal intensity increased with decreasing carbon chain length. After one hour, the fluorescent signal obtained with $C_2$FDG was nearly 1000-fold higher than with $C_{12}$FDG in cultures of *M. smegmatis* and BCG harboring *M. smegmatis gyrB*::*lacZ* plasmids in multicopy. $C_2$FDG also detected gene expression by *M. smegmatis* and BCG that had been internalized by J774.16 murine macrophages. Detection levels were $1x10^5$ for BCG and $2x10^5$ for *M. smegmatis* bacteria with the multicopy plasmid and $3x10^5$ *M. smegmatis* bacteria with the single copy, integrated fusion. This sensitivity is comparable to what we have seen with green fluorescent protein. This assay also works with *Bacillus subtilis*, *Escherichia coli*, and *Yersinia pestis* using *lacZ* fusions to native promoters suggesting that it is applicable to the study of other bacterial pathogens. This system is being used to monitor bacterial gene expression in mycobacteria under various extracellular and intracellular conditions.

# MYCOBACTERIUM AVIUM INVADES INTESTINAL EPITHELIAL CELLS THROUGH THE APICAL MEMBRANE BUT NOT BY THE BASOLATERAL SURFACE.

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*Mycobacterium avium* is a common pathogen in AIDS patients that is chiefly acquired through the gastrointestinal tract, leading to the development of bacteremia and disseminated disease. To cause infection through the gastrointestinal route, binding and invasion of the intestinal epithelial barrier are required. *M. avium* has been shown to colonize the terminal ileum in a mouse model of infection, as well as to bind and invade different human cell lines *in vitro*, including intestinal epithelial cell lines such as HT-29. To further characterize this process and determine the preferential site (basolateral vs apical membrane) of *M. avium* invasion we have infected the human intestinal epithelial cell line Caco-2 with the strain 101 of *M. avium* (MAC101). The levels of binding and invasion of confluent Caco-2 monolayers by MAC101 were similar when the assay was carried out with control medium in presence of $Ca^{++}$ (when only the apical surface is exposed), with $Ca^{++}$-depleted medium, or even with $Ca^{++}$-depleted medium + 1 mM EGTA (exposure of both apical and basolateral membranes). These results suggest that the point of entry is the apical surface of the epithelial lining. Because growth within macrophages has been shown to increase the efficiency of MAC in invading other macrophages we examined if MAC grown within Caco-2 cells would show a similar invasive phenotype. MAC101 was grown within Caco-2 cells for seven days, and then released from the cells and used to infect new monolayers of epithelial cells. Both binding and invasion were significantly greater (between 2 and 5 fold) than those obtained with bacteria grown on plates. In addition, the invasion occurred only by the apical surface. It is plausible that the initial step of multiplication in the intestinal epithelial cells could be relevant *in vivo*, as it would allow the bacteria to express new proteins and infect more efficiently other epithelial cells or macrophages.

In conclusion, we show that invasion occurs through the apical surface and that growth of the bacteria within intestinal epithelial cells results in an increased efficiency of invasiveness. These findings have important implications in the understanding on the mechanisms of *M. avium* pathogenesis.

# A STRUCTURALLY VARIABLE SURFACE PROTEIN OF *ENTEROCOCCUS FAECALIS*, ENRICHED AMONG CLINICAL ISOLATES, IS ENCODED ON A TRANSPOSON-LIKE ELEMENT

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We recently identified a cell-wall associated protein among clinical isolates of *Enterococcus faecalis*, that shows structural similarities to the Rib and C alpha proteins of group B streptococci, and may relate to *E. faecalis* virulence. The gene encoding the *E. faecalis* protein has been sequenced and found to be unusually large at 5.6 kb. The corresponding protein is inferred to be 1873 a.a. with a predicted mass of 202 kDa. The central part of the inferred protein possesses two distinctly different repeat motifs. The first of these, located on the N-terminal side, consists of three tandem 84 a.a. repeat units encoded by very highly conserved 252 bp nucleotide repeats. This is followed by a second repeat motif, consisting of seven complete and one partial 82 a.a. repeat units, encoded by again highly conserved 246 bp nucleotide repeats. Identity with the Rib and C alpha proteins occurs within these latter repeat units and is restricted to a stretch of 11 a.a. that is nearly identical between all three proteins. Among six enterococcal species tested, the presence of this gene was limited to *E. faecalis*. Studies on the expression of this gene in commensal and clinical isolates of *E. faecalis* reveal an enrichment in infection derived isolates suggesting that it contributes to bacterial colonization or survival in disease. Further, analysis of the structural gene among infection derived and commensal isolates reveals variability in the size of this surface protein, corresponding to the addition or deletion of repeat units. This suggests that this *E. faecalis* surface protein may vary under immune selection as observed for the group B streptococcal proteins, as well as those recently characterized on the surface of cell wall-less organisms. Restriction fragment and nucleotide sequence analysis of flanking DNA from isogenic strains suggest that this gene is carried on a mobile element that is ~15-20 kb in size. In addition to encoding a transposase homologous to that for insertion sequence element IS*905*, this element encodes proteins with high degree of similarity to proteins of unknown function encoded by the *L. lactis* transposon Tn*5306*. Together, these data suggest that this transposon-like element may harbor virulence determinants that contribute to enterococcal pathogenesis.

# INTER- AND INTRACELLULAR SIGNAL TRANSDUCTION REGULATES EXTRACELLULAR TOXIN PRODUCTION IN *CLOSTRIDIUM PERFRINGENS.*

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Gram-positive anaerobic pathogen, *Clostridium perfringens* produces a variety of toxins whose synergistic actions play important roles on the pathogenicity (gas gangrene in humans) of this organism.

A two-component regulatory system, VirR/VirS, had been identified as global regulatory system for the toxin production in *C. perfringens*. VirS is a sensor histidine kinase and VirR is the cognate response regulator. Northern analysis showed that the transcription of alpha- (*plc*), theta- (*pfoA*), and kappa- (*colA*) toxin genes was positively regulated by this system. Although primer extension analyses revealed a VirR/VirS-dependent promoter in each toxin gene, the promoter region was totally different from each other, suggesting that more complex regulatory network might exist for the regulation of toxin genes in *C. perfringens*.

In addition to the intracellular signal transduction mediated by the VirR/VirS system, existence of an intercellular signal transduction (cell-cell communication), which governs the production of several toxins, have been suggested. We have isolated a mutant which did not produce several toxins from wild type *C. perfringens* strain 13. This mutant (SI112) only produced toxins when the strain was mixed with the culture supernatant of strain 13. This suggests that wild type *C. perfringens* produces an extracellular signaling substance into the culture supernatant which stimulate toxin production of other cells. We characterized this signal substance using strain SI112 as indicator strain. The transcription of the *pfoA*, *colA*, and *plc* genes significantly increased when SI112 was mixed with strain 13 culture supernatant, indicating that the signaling substance activates the toxin genes at the transcriptional level. Production of this substance from *C. perfringens* mainly seen in the exponential phase and decreased on entering to the stationary phase. Further characterization and purification of this stimulatory substance are under progress.

# FILAMENTOUS GROWTH AND THE VIRULENCE OF *CANDIDA ALBICANS*

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*Candida albicans* is the prevailing cause of invasive systemic fungal infections in immunocompromised people. Upon exposure to host conditions, such as the presence of serum, yeast-form Candida undergo a dramatic concerted conversion to filamentous hyphal growth. The abundance of filamentous forms of this organism in infected tissues suggests that tthis dimorphic transition of Candida may be crucial for virulence. Recent genetic studies of the dimorphic transition in a related model fungus, *Saccharomyces cerevisiae*, has allowed us to identify conserved functions required for Candida dimorphism and to test their roles in virulence directly.

Because *C. albicans* forms invasive filaments by continuously focusing new cell growth to emerging hyphal tips we sought to identify genes involved in this polar growth process. A Candida genomic library was screened for sequences that correct the temperature sensitivity of a Saccharomyces mutant (*cdc24)* having a cell polarity defect. One of the genes isolated from this screen is a homolog of the Saccharomyces *BUD1* gene, which encodes a Ras-related GTPase required for proper selection of growth sites. Using homologous recombination we created a homozygous Candida *bud1* knockout mutant that, like a Saccharomyces *bud1* mutant, grows at a normal rate but places new buds randomly on the cell surface. Strikingly, under conditions that induce filament formation, the Candida mutant produces only bent hyphae that invade an agar substrate very poorly. To test the effect of abnormal hyphal growth on virulence, mice were infected with this mutant. The *bud1* mutant is significantly less virulent than wild type as measured by mouse mortality. Histologic examination demonstrated non-invasive Candida in the kidney tissue of mice injected with the *bud1* mutant. We conclude that filamentous growth is a virulence factor for invasive infection by *Candida albicans* . Therefore, regulators of fungal cell polarity are potential antifungal drug targets.

## COMPARISON OF DIFFERENT *HELICOBACTER PYLORI* ANTIGENS ISOLATED FROM PATIENTS WITH DUODENAL ULCER AND ASYMPTOMATIC CARRIERS

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*Aim:* The present study was done to determine the presence and relative quantities of different antigens/virulence markers in *H. pylori* strains isolated from patients with duodenal ulcer (DU) and from asymptomatic carriers (AS).

*Methods: H. pylori* strains were isolated from antral biopsies of 21 patients with DU (14 males, mean age 54) and 20 AS carriers (16 males, mean age 50). The presence of HpaA, flagellin, urease and 26 kD proteins, Lewis sugar epitopes, vacuolating cytotoxin A (VacA) and the cytotoxin-associated gene A (*cagA*) were determined by whole bacteria coated ELISAs, tissue culture test or PCR. We also determined quantitative differences of various antigens by inhibition-ELISAs using specific monoclonal antibodies.

*Results:* HpaA, flagellin, urease and 26 kD proteins were present in all *H. pylori* strains from DU and AS subjects. However, quantitative differences in the amount of flagellin, urease and 26 kD proteins were found between different strains, both in DU and AS subjects. Individual Lewis carbohydrates, $Le^x$, $Le^y$, $Le^a$ and $Le^b$ were expressed in 52, 71, 19 and 24% respectively of the DU strains compared to 40, 75, 0 and 10% respectively of the AS strains. The presence of the most common sugar combination, $Le^x$ and $Le^y$ were roughly 40% of both strain categories and $Le^a$ and $Le^b$ were coexpressed in less than 10% of the DU strains. Only on very rare occations more than 2 Lewis structures were exposed on the surface of the DU strains. Strains isolated from DU and AS carriers never expressed $Le^x$ or $Le^a$ structures alone. However, 5% of DU and AS strains were strictly $Le^b$ positive. Further, 14% of DU strains and 35% of AS exclusively expressed $Le^y$ structures. None of the strains elicit sialyl-$Le^x$ structures. Fourteen and 20% of the DU and AS strains respectively were non-typable for any Lewis epitope. Approximately 85% of the strains contained the cagA gene in the respective study group. Forty three percent of *H. pylori* strains from the DU patients and 35% of the strains from AS expressed the cytotoxin.

*Conclusion:* In conclusion we found no significant differences in qualitative expression of any of the antigen markers studied in DU and AS subjects. However, there were substantial differences in the amount of antigens, e.g flagellin, urease and the 26 kD protein, between different isolates.

# A MOUSE MODEL OF INTRANASAL BRUCELLA MELITENSIS INFECTION AND PROTECTION WITH AN INTRANASAL LIPOPOLYSACCHARIDE VACCINE

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Brucella are intracellular bacterial pathogens that cause abortion in animals and chronic relapsing disease in humans. Infection typically occurs via a mucosal route such as the conjunctiva, respiratory tract or intestinal tract. A mouse intranasal infection model showed that following inoculation Brucella melitensis 16M migrate to the spleen and other organs, mimicking the course of natural infection. Inoculation of $10^1$, $10^3$, $10^4$ and $10^5$ cfu resulted in infection rates of 0%, 50%, 90% and 100%, respectively. Inflammatory cytokines (TNFα, IL6) were not detected in lungs after inoculation. Infection was documented by culture of bacteria from the spleen. In situ bacteria were detected by immunohistochemistry. To test the protective potential of local immunization, a vaccine consisting of the lipopolysaccharide (LPS) of B. melitensis 16M hydrophobically complexed with the outer membrane protein (OMP) of Group b Neisseria meninigitidis was used to immunize mice by the intranasal route. Two doses of the LPS + OMP vaccine induced high levels of pulmonary IgA and IgG, and serum IgG, specific for 16M LPS. Secretory IgA bound bacteria in vitro. Efficacy of the vaccination regimen was tested by challenging immunized and control (saline treated) groups of mice intranasally with $10^4$ cfu of 16M at 2 wks or 6 wks after boosting. Spleen homogenates were cultured for quantitation of 16M at 8 wks post challenge. In mice challenged at 2 wks, positive spleen cultures were found in 5/15 immunized mice vs 12/15 control mice ($p = 0.025$) and the mean cfu in infected spleens of immunized mice were significantly lower ($p = .01$). Likewise, in mice challenged at 6 wks, 1/15 immunized mice vs 8/15 control mice ($p = .014$) were infected.

# STUDIES ON INTERACTION BETWEEN IgG-BINDING UNITS DERIVED FROM PROTEIN MIG AND GOAT IgG SUBCLASSES

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The α-haemolytic group C streptococci isolated from mastitis (*S. dysgalactiae*) have numerous surface proteins, which specifically interact with certain plasma or connective tissue proteins of the host. Some of these proteins bind to immunoglobulin G (IgG) of various animal species in a non-immune way via the constant region of the IgG molecule, which might interfere in various ways with the recognition of the bacteria by the host immune system.

Protein MIG is the IgG-binding surface protein from *S. dysgalactiae* strain SC1. The gene coding for protein MIG has been cloned and sequenced in our laboratory (Jonsson and Müller, Eur.J.Biochem. 220:819-826, 1994). The N-terminal region of protein MIG specifically binds to the fast form of the proteinase inhibitor $\alpha_2$-macroglobulin. This region is directly followed by five, 70 aa long, highly similar repeat units, which mediate binding to IgG. The presence of five IgG-binding units is so far unique for protein MIG in comparison to the general 2 or 3 repeats found in similar Fc-receptors of group C and G streptococci.

Various DNA fragments of the region, coding for the IgG-binding activity in the *mig* gene, were ligated into the expression vector pMALc2, so that the different constructs contained increasing number of the repeats, starting from the most N-terminal unit. The corresponding proteins were expressed in *E. coli* as fusions to the maltose-binding protein and affinity purified on amylose-resins. The interactions of these constructs with goat IgG subclasses were studied using conventional ELISA and RIA techniques. The binding of the five-repeat molecule, mimicking the native receptor, to Fc and Fab fragments of goat IgG was tested as well.

All protein MIG derived constructs bound both subclasses of goat IgG. The increase in number of IgG-binding repeats enhanced the affinity between the receptor molecule and the IgG. The five-repeat construct reacted with both Fc and Fab fragments of goat IgG. The presence of five IgG-binding units in protein MIG, its capacity to bind to both Fc and Fab fragments of the immunoglobulin and the revealed high avidity to IgG might indicate, that the IgG-receptor complex is stabilised by multiple-point interactions between the molecules.

THE ROLE OF CD14 IN SIGNALING MEDIATED BY OUTER MEMBRANE LIPOPROTEINS OF *BORRELIA BURGDORFERI*. R. Mark Wooten, Tom B. Morrison, John H. Weis, Rolf Thieringer, Samuel D. Wright, and Janis J. Weis. University of Utah School of Medicine and Merck Research Laboratories.

*Borrelia burgdorferi* possess membrane lipoproteins which exhibit stimulatory properties and, consequently, have been implicated as playing a role in the pathology seen in Lyme disease. As CD14 has been shown to mediate signaling by a number of lipid-modified bacterial products, we wanted to determine if CD14 played a role in signaling mediated by *B. burgdorferi* lipoproteins (OspA and OspC) in human umbilical vein endothelial cells (HUVEC). In the absence of serum (thus no CD14), lipoproteins were able to induce HUVEC to translocate the transcription factor, NF-kB, to the nucleus, as well as secrete IL-8 and IL-6. However, the addition of rCD14 increased the sensitivity of HUVEC to OspA and OspC 10- to 100-fold, while a rCD14 lacking the LPS-binding site had no such effect. Antibodies which have been shown to bind CD14 and inhibit LPS-mediated signaling were also able to inhibit lipoprotein signaling. Western blot analyses were performed to determine if OspA was physically interacting with CD14. OspA did form a complex with CD14, as seen by band shifts on native gels, but did not interact with rCD14 lacking the LPS-binding site. Titration studies indicated that LPS can compete with OspA-CD14 complexes, suggesting that LPS and OspA bind similar portions of the CD14 molecule. The binding of OspA to CD14 appears to be lipid-mediated, as $Pam_3Cys$ (a synthetic lipopeptide similar to the amino terminal portion of OspA) can compete with OspA- or LPS-binding to CD14, while a rOspA lacking the lipid modification was unable to compete. Altogether, these findings indicate that CD14 can help mediate lipoprotein signaling, but it is not the ligand-specific receptor responsible for activating lipoprotein-sensitive cells.

# ANTIMICROBIAL ACTIVITY AND APOPTOTIC POTENTIAL OF CATHELICIDIN-DERIVED PEPTIDES OF INNATE IMMUNITY

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Antimicrobial peptides are an ancient component of innate immunity and provide a first line of defense against invading microorganisms. In mammals they are found in epithelia and in professional phagocytes. A variety of these peptides are derived from precursors of the cathelicidin family first identified in neutrophils. Members of this family show a highly conserved preproregion and a C-terminal domain that exhibits antimicrobial activity once cleaved from the precursor. The properties of some of these peptides have been investigated by using synthetic peptides corresponding to the antimicrobial sequences. We show here data on the *in vitro* antimicrobial activity of a novel cathelicidin-derived peptide of 34 residues (BMAP-34) deduced from a bovine gene sequence, and the apoptotic potential of two known bovine myeloid members of 27 and 28 residues (BMAP-27 and BMAP-28). All these peptides are cationic and show an amphipathic α-helical conformation. The spectrum of activity of BMAP-34 includes Gram negative (e.g. *Escherichia coli, Serratia marcescens*, *Salmonella typhimurium* and *enteriditis*) and Gram positive (e.g. *S. epidermidis, B. megaterium,* several strains of *S. aureus*, including methicillin-resistant strains, MRSA) bacteria, with MIC values in the 1-12 μM range. The peptide is active against *Cryptococcus neoformans* (MIC of 3 μM) but not against *C. albicans* nor does it show lytic activity on human red blood cells even at concentrations much higher than those antibacterial (<2% hemolysis at 50 μM peptide).

BMAP-27 and -28 are known antimicrobial peptides. We have found that they exert a low toxicity on bovine or human peripheral blood leukocyte populations including resting lymphocytes. However, proliferating cells like tumor cell lines and *in vitro* activated normal lymphocytes are permeabilized by micromolar concentrations of peptides. The permeabilization is followed by apoptotic death, as indicated by the typical DNA fragmentation and morphologocal changes. This effect is exerted on Pha and rhIL2 stimulated lymphocytes over a culture period of 30 days. These peptides may thus be effector molecules of the defense response mediated by neutrophils, and may also contribute to modulate the immune response at inflammation sites by clearing activated lymphocytes and thereby preventing the debilitating effects of chronic inflammation. As these are primarily mediated by activated T lymphocytes, which cause cellular infiltration and tissue damage through cytokine production, the apoptosis mediated by the BMAP peptides may be one of the mechanisms used to break the unrelenting cellular activation.

# INTERACTION OF THE MYCOBACTERIAL PHAGOSOME WITH THE HOST ENDOCYTIC PATHWAY

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*Mycobacterium tuberculosis* parasitizes host macrophages and resides in a phagosome that resists acidification and fusion with lysosomes. Compared with inert phagocytic targets, the *M. tuberculosis* phagosome exhibits maturational arrest manifested by persistence of plasma membrane proteins and early endocytic markers, a persistent capacity to interact with early endosomes and acquire exogenously added transferrin, and a limited acquisition of lysosomal markers such as lysosomal membrane glycoproteins and cathepsin D. We have made similar observations on the *M. leprae* phagosome in studies of skin biopsy samples from subjects with lepromatous leprosy. These results contrast sharply with our observations of another intracellular pathogen, *Legionella pneumophila*, which also resists acidification and fusion with lysosomes. In contrast to our findings with *M. tuberculosis* phagosomes, mature *L. pneumophila* phagosomes lack all markers of the endosomal-lysosomal pathway studied to date.

We have examined the trafficking of mycobacterial antigens secreted or shed from the mycobacteria into their phagosomes, including the 30 - 32 kDa mycolyl transferases and mycobacterial lipo-arabinomannan (LAM). We have found that these mycobacterial antigens do traffick through the endosomal-lysosomal pathway. In addition, we have found that mycobacterial LAM trafficks to the specialized MIIC compartment. Thus, although the *M. tuberculosis* and *M. leprae* phagosomes exhibit arrested maturation along the endosomal-lysosomal pathway, these phagosomes interact with the endocytic pathway in a bidirectional fashion, receiving material from and delivering material to the endocytic pathway.

# INHIBITION OF INDUCIBLE NITRIC OXIDE SYNTHASE ACTIVITY RESULTS IN REACTIVATION OF LATENT TUBERCULOSIS IN MICE

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A unique feature of *Mycobacterium tuberculosis* is its ability to establish latent infection in the human host, which can reactivate to cause disease years later. The mechanisms involved in the control of latent tuberculosis were examined using two murine experimenal tuberculosis models. A low-dose *M. tuberculosis* infection that is maintained at a stable level for many months was the first model tested. Treatment of mice infected six months previously with aminoguanidine (AG), an inhibitor of inducible nitric oxide synthase (iNOS), resulted in reactivation of *M. tuberculosis*, as exhibited by hepatosplenomegaly, a robust tissue granulomatous reaction, and greatly increased numbers of bacilli in the lungs. IFN-$\gamma$, TNF-$\alpha$, and iNOS were shown to be expressed throughout the latent phase of infection, and increased upon reactivation. Reactivation of latent tuberculous infection by inhibition of iNOS was confirmed using a second murine tuberculosis model, which is based on treatment with anti-mycobacterial drugs. Results obtained using this drug-based model also suggested the existence of reactive nitrogen intermediate (RNI)-independent mechanisms of control during the latent phase of infection. Thus, our results suggest that both RNI-dependent and RNI-independent mechanisms contribute to the prevention of tuberculous reactivation.

# A NOVEL MACROPHAGE INVASION MECHANISM OF PATHOGENIC MYCOBACTERIA REQUIRES COMPLEMENT COMPONENT C2a

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Mycobacteria are intracellular pathogens which reside almost exclusively within monocytes/macrophages in infected individuals. Experimental results have indicated that monocytes and macrophages are critical for controlling the initial stages of infection and for granuloma formation. Further, macrophages serve as the reservoir for mycobacteria in disseminated infections. Therefore, the ability of monocytes and macrophages to phagocytose the mycobacteria is pivotal for mycobacterial survival and for the host immune response.

We have purified a protein from heat treated equine and human serum which markedly enhances invasion of macrophages by pathogenic mycobacteria (*M. leprae, M. avium, M. tuberculosis* and BCG) but has no effect on invasion by fast growing non-pathogenic mycobacteria (*M. vaccae, M.smegmatis* and *M. Phlei*). Further, other intracellular pathogens including *Nocardia asteroides, Listeria monocytogenes* and *Leishmania mexicana* could not use this serum factor to mediate macrophage entry. Sequence analysis of the purified equine 70 KDa protein revealed that its amino terminus was identical to the N-terminal sequence of human C2a in 19 of 20 amino acids. Our findings were unexpected since C2a has been shown to function only as part of the complement C3 cleaving enzyme (convertase), C4b2a. To confirm C2a as the active component, fractionated protein from heat treated equine and human serum were depleted of C2a using a polyclonal antibody to human C2. These fractionated serums depleted of C2a were unable to mediate *M. avium* uptake. Moreover, C2a generated by incubation of purified C1, C2 and C4 with IgM coated erythrocytes enhanced *M. avium* invasion of macrophages. Subsequent analysis revealed that the serine protease activity of the C2a was required, and that C2a in association with *M. avium* could cleave C3 and lead to C3b deposition on the mycobacteria. This unique C3 convertase could also cleave C3 secreted by macrophages and lead to C3 deposition on the mycobacteria, as shown by the inhibition of invasion using anti-C3 receptor monoclonal antibodies and through the failure of C2a to induce *M. avium* invasion of C3-deficient macrophages. Therefore, pathogenic mycobacteria have evolved a mechanism to salvage C2a and use it as a means of invading macrophages. Significantly, C2a does not reassociate with C4b and thus in the absence of these pathogenic mycobacteria, has no ability to cleave C3. This unique invasion process has the characteristics of a virulence mechanism important for mycobacterial pathogenesis.

NONOPSONIC BINDING OF *MYCOBACTERIUM TUBERCULOSIS* TO COMPLEMENT RECEPTOR TYPE 3 IS MEDIATED BY CAPSULAR POLYSACCHARIDES AND IS STRAIN DEPENDENT

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The establishment of an intracellular infection is a key feature of the pathogenesis of tuberculosis. The choice of host cell receptor and the mechanism of binding (opsonic versus nonopsonic) may influence the intracellular fate of *Mycobacterium tuberculosis* (*M.tb.*). We have identified two substrains of *M.tb.* H37Rv, designated H37Rv-CC and -HH, that differ in their mode of binding to complement receptor type 3 (CR3) expressed in transfected Chinese hamster ovary (CHO-CR3) cells: H37Rv-CC binds nonopsonically, whereas H37Rv-HH only binds after opsonization in fresh serum. CR3 is an important monocyte/macrophage receptor; nonopsonic binding could provide a critical advantage in the complement-poor alveolar space. H37Rv-CC also binds nonopsonically to untransfected CHO cells, whereas H37Rv-HH binding is enhanced by serum and is mediated by 1D1 antigen, a bacterial adhesin previously identified as a polar phosphatidylinositol mannoside (PIM). H37Rv-CC and -HH have identical 1S6110 fingerprint patterns. Of 5 *M.tb.* clinical isolates examined, 4 display the same binding phenotype as H37Rv-CC, as does the Erdman strain, whereas 1 isolate, as well as *M. smegmatis*, behaves like H37Rv-HH. Nonopsonic binding of H37Rv-CC to CHO cell-expressed CR3 is apparently to the β-glucan lectin site, and it is inhibited by removal of outer capsular polysaccharides, as well as by the presence of capsular D-glucan and D-mannan from *M.tb.* Erdman, but not by Erdman D-arabino-D-mannan, yeast mannan, or capsular components from H37Rv-HH. Thus, outer capsular polysaccharides appear to mediate the binding of H37Rv-CC to the CR3 lectin site. We propose there are strain-dependent differences in thickness or composition of capsular polysaccharides that determine the mode of binding of *M.tb.* to mammalian cell receptors. These differences may correlate with virulence and may dictate the outcome of an infection.

CORRELATION BETWEEN THE METABOLIC ACTIVITY OF *MYCOBACTERIUM* AND THE ACCESSIBILITY OF ITS VACUOLE TO THE ENDOCYTIC NETWORK

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It has previously been reported that *Mycobacterium tuberculosis* and *Mycobacterium avium* phagosomes fail to accumulate endocytic markers, such as dextran or bovine serum albumin, yet they remain in communication with the early recycling pathway of the host macrophage as demonstrated by the continuous entry of transferrin. We are currently developing a "capture assay" to ascertain whether endocytosed material passes through the pathogen phagosome, indicative of a compartment with access to sorting endosomes. *M. avium* are biotinylated via their surface proteins and allowed to infect bone-marrow macrophages for varying times. After internalization is complete, the macrophages are given fluorescently-labeled streptavidin as an endocytic marker; if this marker passes through the *M. avium* phagosome, it should label the surface of the biotinylated bacteria. The *M. avium* are then isolated from the phagocytes, and the viability stain fluorescein diacetate (FDA) used to determine the metabolic state of the bacteria. In preliminary experiments, the bacteria which acquire the stretavidin are NOT metabolically active, i.e. fail to stain with FDA. This suggests that *M. avium* with high metabolic rates maintain vacuoles which avoid interaction with vesicles destined for lysosomes, while bacteria in stasis may reside in vacuoles with the characteristics of sorting endosomes or even later compartments along the lysosomal pathway. The results of these experiments have implications for the survival mechanisms utilized by *Mycobacterium*, the intersection of the bacteria with the host-cell's antigen processing and presentation machinery, and the access of anti-microbial agents to these intracellular pathogens.

*Mycobacterium tuberculosis erp* ENCODES A REPETITIVE, SURFACE-ASSOCIATED PROTEIN REQUIRED FOR PERSITENCE WITHIN THE HOST.

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Using the *phoA* reporter gene for the systematic search of *M.tuberculosis* DNA sequence encoding exported proteins, we recently identified and cloned the *erp* gene. The distribution of *erp* is restricted to major mycobacterial pathogens of the *M. tuberculosis* complex and to *M. leprae*. It encodes an Exported Repetitive Protein (ERP) containing a typical signal sequence and 12 degenerate repeats of the PGLTS motif. Recombinant forms of ERP were produced in *Escherichia coli* , affinity purified and used to raise anti-ERP rabbit sera. These sera detected a 36-34 kDa protein doublet in *M. tuberculosis* culture filtrates. Surface analysis by immunoelectron microscopy revealed intense gold labeling at the periphery of bacilli. Additionally, ERP was detected in the phagosomal compartment of J774 murine macrophages infected with *M. tuberculosis*. To investigate the contribution of *erp* during infection by members of the *M. tuberculosis* complex, a null *erp*- mutant was constructed in *M. bovis* BCG by allelic exchange . The resulting *erp*- mutant strain presented no obvious phenotype *in vitro* and its multiplication was not affected under axenic conditions. However, persistence of the *erp*- BCG mutant strain in mice was severly impaired when compared to wild type BCG. This difference was exacerbated in the lungs, which represent the primary site of infection by the bacteria of the *M. tuberculosis* complex. These data support that ERP is a surface component of *M. tuberculosis* that may be essential for persistence/virulence within the host.

MICROBIAL GENOMES: FROM SEQUENCING TO COMPUTER AND FUNCTIONAL ANALYSIS. J.-F. Tomb, O. White, R.A. Clayton, A. R. Kerlavage, H. P. Klenk , R. D. Fleischmann, K. A. Ketchum, G. G. Sutton, B. A. Dougherty, K. Nelson, S. Gill, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, K. McKenney, P. D. Karp, M. D. Adams, H. O. Smith, C. M. Fraser, J. C. Venter. The Institute For Genomic Research, 9712 Medical Center Drive, Rockville, MD 20850 USA.

It has become clear that the availability of complete microbial genome sequences profoundly affects our ability to address biological questions. Not only does this information provide new insights into genome organization, overall gene content, and regulation of gene expression, but it also opens the door to discovery of novel genes, gene families, and biochemical pathways in pathogens and organisms of potential industrial importance. Moreover, these data underscore how little we actually know about microbial species. Each completed genome analysis reveals novel sets of genes (many of which may be unique to a particular species) and an astonishing complexity and plasticity in even the smallest genomes. Given that microbes account for the bulk of the biomass on this planet, are found in the most extreme environments, and play a critical role in overall global ecology, it is clear that the species which have been subjected to whole genome analysis to date represent only the "tip of the iceberg" of biodiversity at the molecular level. In addition to its utility for new gene discovery, the increasing number of whole genome sequences will shed new light on evolutionary relationships among species outside of the context of rRNA sequences. The presence of a large number of proteins with no known functions (~ 40 % of the proteins of every sequenced genome) poses a challenge to both experimental and computer scientists. Improved methods of analysis and annotation of microbial genomes are being developed. They include sequence-based strategies (e.g., pairwise alignement for gene identification or more sensitive methods including the use of HMM protein families and specialty composition algorithms such as those that measure propensity for signal peptide and membrane spanning domains), structural classification methods, phylogenetic and metabolic pathway tools. Strategies for global expression and functional analyses are being refined and include DNA and protein microarraying technologies.

# DEVELOPMENT OF NOVEL VACCINES AGAINST INFECTION WITH *HELICOBACTER PYLORI.*

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Infection with the gastroduodenal pathogen *Helicobacter pylori* is now established as the etiologic agent of chronic gastritis and most cases of peptic ulcer disease worldwide. Recent evidence linking infection to gastric adenocarcinoma and non-Hodgkin's lymphoma, has resulted in classification of this bacterium as a "Class I" carcinogen by the WHO. An immunization strategy for the prevention of infection would significantly reduce the risks for development of peptic ulcer disease and gastric cancer.

A preventative approach employing active immunization is now an established method in animal models for studying efficacy of helicobacter antigens. Early studies identified both recombinant *H. pylori* urease apoenzyme and a GroES homologue to protect from infection in a heterologous model of helicobacter infection. More recently, the *in-vivo* adaptation of *H. pylori* in mice has allowed for efficacy studies with species-specific antigens as potential vaccine candidates. This has enabled the screening of novel genes encoding membrane proteins, identified through transposon shuttle mutagenesis, in an *H. pylori* murine model of infection.

The complete sequencing of the *H. pylori* genome will provide important information about the pathogenic mechanisms associated with infection and disease, and will significantly influence studies in areas of both immunoprophylaxis and rational drug design. We have sought to utilize this information to identify suitable novel vaccine candidates for study, and will present preliminary data from analysis of the genome in search for new Helicobacter vaccines.

## PROTEOMICS: APPLICATIONS IN MICROBIAL GENOMICS.

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The introduction of commercially available fixed pH gradient gels for the first dimension of 2D-PAGE has increased the reproducibility between research groups as well as within the laboratory. Improvements in matrix-assisted laser disruption ionisation mass spectrometry (MALDI-MS) together with the great increase in genomic DNA sequences deposited in the databanks have facilitated the rapid identification of polypeptides and have dramatically transformed the usefulness of this technology. The major limitation is the number of spots that can be visualised with coomassie blue staining, for this determines those spots which can be excised for polypeptide identification.

Presently the location of about 330 of the potential 1703 polypeptides of *Haemophilus influenzae* has been placed within the proteome map. Many of these spots were localised and the relative polypeptide identified after enrichment with column chromatography. The number of polypeptides that have the appropriate properties required for the migration within the limits imposed by the pH gradient also dictates a maximum number of proteins that can be examined by this methodology.

For analytical gels $^{35}S$ labelled methionine has been used which allows for the visualisation of approximately 600 spots with a PhosphorImager. Accurate quantification of intensities is hindered by the lack of reproducibility between gels such that at least five gels are required for each experimental point for the statistical evaluation of the spot intensities. We are using this methodology to examine the regulatory networks induced by various classes of antibiotics in an attempt to gain insight into the mechanisms of older anti-microbials with either a poorly or un- defined mode of action. Induction with four classes of antibiotics has been shown to result in a pattern of expression that is consistent with the inhibition of the respective target molecule(s).

Initial experiments revealed that mRNA and protein synthesis were qualitatively but not quantitatively correlated. Experiments to examine the importance of translational control, using conventional Northern blots, are in progress, however, the newer chip technologies to determine the levels of mRNA are also being evaluated.

WHOLE GENOME ANALYSIS USING HIGH-DENSITY ARRAYS

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As we enter the post genome era, one of the main challenges facing researchers is to determine the biological function of thousand of ORFs discovered by the genome sequencing projects. We have developed a high-throughput approach for generating and analyzing deletion strains in the budding yeast S. *cerevisiae* [Shoemaker et al (1996) Nature Genetics]. A PCR-based targeting strategy is used to generate deletion strains that are labelled with unique molecular tags (20mers) that can be detected by hybridization high-density arrays containing oligo-nucleotides complementary to the 20mer sequences. The tags allow large numbers deletion strains to be pooled and processed in parallel through selective growth assays. Our goal is to identify phenotypes for as many of the mutants as possible and use this information to help elucidate the biological function of the thousand of uncharacterized genes. An international consortium has been assembled to generate tagged deletion strains for each of the ~ 6,000 ORFs in the yeast genome.

Many of the whole genome analysis techniques that have been developed for yeast can be applied to other organisms once their genomes are sequenced. Several examples involving microbial pathogenesis will be discussed.

# REGULATED EXPRESSION OF A MULTIGENE FAMILY OF ANTIGENIC PROTEINS IN *BORRELIA BURGDORFERI*

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Lyme disease is caused by the spirochete *Borrelia burgdorferi* and transmitted by the bite of infected *Ixodes* ticks. Similar to what has been observed with some other arthropod-borne bacteria, *B. burgdorferi* alter the expression of their surface proteins in response to a shift of culture temperature from 23°C to 35°C, a change mimicking that which occurs upon the ingestion of a blood meal by the tick. We have identified a family of 12 closely related proteins, designated Erps, that are upregulated during such a temperature shift. Animal studies indicate that the antigenic Erp proteins are expressed early in mammalian infection and may function in the transmission from the tick vector into the warm-blooded host.

The genes encoding the Erp proteins are arranged in 8 loci, each of which is located on a separate plasmid having characteristics suggestive of a bacteriophage genome. Comparisons of *erp* gene sequences from different isolates of *B. burgdorferi* indicate considerable variation, possibly the results of past recombination events. Exchange of genetic material encoding early antigens such as the Erp proteins may then permit these bacteria to re-infect a previously exposed mammal.

One of the major obstacles to the study of spirochetes such as *B. burgdorferi* is the lack of genetic tools. We are therefore studying the *B. burgdorferi* plasmids that carry the *erp* genes for possible uses as shuttle vectors or transducing phages.

## ANALYSIS OF THE BOUNDARIES AND DISTRUBUTION OF *SALMONELLA* PATHOGENICITY ISLAND 2 (SPI2)

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Two chromosomal pathogenicity islands have been identified in *Salmonella typhimurium*. *Salmonella* pathogenicity island 1 (SPI1) at 63 centisomes harbors invasion genes involved in epithelial cell entry. The second locus SPI2 is located between 30 and 31 centisomes on the *S. typhimurium* chromosome. Inactivation of SPI2 genes by transposon insertions resulted in $10^4$-fold attenuation of virulence in the murine salmonellosis model (1). SPI2 mutants were not deficient in cultured epithelial cell entry or survival in cultured macrophages (2). However, these mutants failed to proliferate in host organs like liver and spleen of infected animals after oral, intraperitoneal or intraveneous infection. Thus a function of SPI2 during the late stages of infection by *S. typhimurium* was concluded. SPI2 contains genes encoding a type III secretion system whose function is distinct from that of the type III secretion system encoded by SPI1 (2).

An analysis of the boundaries of SPI2 and comparison with the corresponding region of the *Escherichia coli* chromosome revealed that SPI2 consists of a DNA element of about 40 kb specific to *S. typhimurium* that is inserted adjacent to the $tRNA^{val}$ gene. At the region corresponding to the SPI2 insertion point the *E. coli* chromosome contains 9 kb of DNA which appears not to be present in *S. typhimurium* (3).

The distribution of SPI1 and SPI2 was examined in various *Salmonella* isolates. In contrast to type III secretion system genes of SPI1, those of SPI2 are not present in *S. bongori*, which diverged at the first branch point in the *Salmonella* lineage. This and other data indicate that SPI2 was acquired by a *Salmonella* strain already harboring SPI1 by horizontal transfer from an unknown source (3).

We present further analysis of the genomic organization of SPI2 in *S. typhimurium* and detailed comparison to the corresponding genomic region in *S. bongori* and *E. coli*. In contrast to the organization of SPI1 a complex mosaic structure at the insertion point of SPI2 was observed. Mutagenesis of newly identified genes in SPI2 and virulence analysis was performed in order to reveal the role of these genes in the context of SPI2 function.

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Use of interspecies recombination for in vivo genomics. Thomas Zahrt, and Stanley Maloy. Department of Microbiology, University of Illinois, Urbana, IL 61801

Closely related species of bacteria often cause different diseases or have different host specificities. For example, although *Salmonella typhimurium* and *S. typhi* are about 98% identical at the DNA sequence level, *S. typhimurium* causes diseases of varying severity in a wide variety of hosts while *S.typhi* causes a severe systemic disease restricted to humans. Construction of genetic hybrids between such closely related species would provide a simple way of testing the in vivo role of defined genomic fragments. The *Salmonella* phage P22 can readily transduce plasmids or transposons from *S. typhimurium* to *S. typhi*; however, inheritance of chromosomal DNA is typically $10^6$-fold less efficient than inheritance of chromosomal DNA between strains of *S. typhimurium*. The length of DNA inherited is also substantially less than that observed during homologous recombination between strains of *S. typhimurium*. Two mechanisms are responsible for this barrier to transduction between *S. typhimurium* and *S. typhi*. First, the frequency of transduction from *S. typhimurium* to mutants of *S. typhi* lacking MutS or MutL is increased by $10^3$-fold, indicating that enzymes involved in mismatch repair act as a barrier to inhibit "homeologous" recombination between these closely related species. Second, the frequency of transduction into derivatives of *S. typhi* lacking MutS and RecD is increased an additional $10^2$-fold, indicating that RecD also acts as a barrier to homeologous recombination. These results indicate that recombination between slightly divergent DNA is much less efficient than recombination between homologous DNA, and the major barrier to recombination between *S. typhimurium* and *S. typhi* is due to the *mutSL* and *recD* gene products.

Inactivating the *mutS* and *recD* genes in the recipient allows efficient recombination between corresponding DNA sequences of *S. typhimurium* and *S. typhi*. This approach can be used to construct hybrids in which small genomic fragments have been substituted between two species, thus allowing direct comparisons of the roles of particular loci. In addition, because the join points of chromosomal insertions or inversions result in linkage disruption, this approach can be used as a simple alternative to pulsed field gel electrophoresis for the rapid comparison of genomic organization.

The Virulence of *Candida albicans* is dependent upon a Dual Morphogenetic Switch Gerald R. Fink Whitehead Institute/M.I.T. , Nine Cambridge Center, Cambridge, MA 02142

*Candida albicans*, switches from a yeast form into two distinct filamentous forms, pseudohyphae and hyphae. This switch is induced by many environmental cues including serum and ingestion by macrophages. Our discovery of a filamentous stage in *Saccharomyces cerevisiae* (induced by nitrogen starvation) encouraged the assumption that morphogenesis in all fungi might be controlled by similar pathways, despite differences in the inductive signals and the shape and form of the filaments. In *Saccharomyces*, the STE20, STE7 and STE12 genes of the mating MAPK pathway are partially responsible for the control of filamentous growth. *Candida* homologs of the STE20, STE7 and STE12 genes were cloned and used to construct null mutations in *Candida*. Strains of *C. albicans* homozygous for mutations in the STE20, STE7 and STE12 homologs though partially defective in hyphal development still form hyphae in response to serum and are virulent, suggesting that there is a second, Ste12p independent pathway by which serum induces filaments in *Candida*.

We used *Saccharomyces* to identify the Ste12p independent pathway in *Candida*. In *S. cerevisiae*, null Ste- mutants are only partially defective for their pseudohyphal defect. The gene responsible for this residual activity in Ste- strains is PHD1 , whose overexpression was shown previously to enhance pseudohyphal growth. Double mutant strains which contain a deletion of the PHD1 gene as well as the Ste- mutation (e.g. ste12/ste12 phd1/phd1) are completely defective in pseudohyphal growth. Recently, EFG1, the *Candida* homolog of the *Saccharomyces* PHD1 gene, was cloned (Stoldt, 1997). Although reduced expression of EFG1 suppressed formation of true hyphae, pseudohyphal filaments were still formed in response to serum. Based on this observation, we constructed *C. albicans*. double mutants lacking both CPH1 and EGF1 function. These *Candida* cph1/cph1 efg1/efg1 strains fail to form filaments in response to serum or other known inducers of filamentous growth and are avirulent in a mouse model.

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CLONING AND CHARACTERIZATION OF AN ADHESIN MEDIATING ADHERENCE OF *CANDIDA GLABRATA* TO EPITHELIAL CELLS.

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We are interested in how pathogenic fungi are able to colonize, persist, and cause disease in the mammalian host. We have been studying *Candida glabrata*, an opportunistic yeast pathogen responsible for significant human disease. *C. glabrata* is haploid, unlike other medically important *Candida* species, raising the possibility that this species might be amenable to genetic analysis. We have established a number of genetic tools in *C.glabrata*: 1) we constructed a ura3 auxotrophic strain that is isogenic with a virulent clinical isolate; 2) we established a system of insertional mutagenesis based on the observation that linear DNA will insert randomly into the genome of *C. glabrata*; 3) we used oligonucleotide tags to mark individual mutants, allowing pools of 96 mutants to be screened at once for any particular phenotype of interest.

We have used these genetic tools to identify mutants of *C. glabrata* that are altered in their ability to adhere to cultured human epithelial cells (Hep2 cells). From an initial screen of 5000 random insertion mutants, we isolated 5 that adhere 2-3 fold better than wild-type, and 16 that adhere approximately 5% as well as wild-type. 14 of these 16 mutants were distributed over a 1.5 Kb region of a single locus. The gene identified by these insertions (EPithelial Adhesin 1 or EPA1) encodes a protein with homology to a large family of yeast cell wall proteins. A precise deletion within the coding region of EPA1 renders the *C. glabrata* non adherent; in addition, expression of EPA1 in *Saccharomyces cerevisiae*, which is normally non-adherent, allows it to adhere to epithelial cells as efficiently as *C. glabrata*. Thus, EPA1 is not only necessary, but probably also sufficient for adherence to epithelial cells, making it likely that EPA1 directly mediates the adherence.

EPA1 mediated adherence to epithelial cells requires Ca++, and can be blocked by as little as 2 mM lactose, but not by a wide variety of other saccharides or glycoconjugates. *C. glabrata* or *S. cerevisiae* expressing EPA1 will adhere to a variety of other cell types, including fibrobloasts and endothelial cells, making it likely that the ligand for the Epa1p is widely distributed among different host cell types. Thus, we have identified a C-type lectin that mediates adherence of *C. glabrata* to a variety of host cell types, likely by binding to a lactosyl-containing host oligosaccharide. We are currently assessing the role of this lectin in pathogenesis. We are also examining the expression pattern of EPA1 to determine what, if any, host signals the yeast is responding to.

# A DNA ELEMENT THAT CONFERS STABILITY DURING MITOSIS IN *CANDIDA ALBICANS*

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The Candida Genome Project is designed to find new genes in the opportunistic human pathogenic fungus *Candida albicans.* To investigate these genes a mitotically stable vector is an essential tool. In this study a DNA fragment that confers stability on linear vectors was found in one of the fosmid clones that comprise the contig map of chromosome 7 in *C. albicans.*

Two selectable telomere-containing constructs were prepared from *Candida albicans* DNA: telomere-*URA3*-ARS and *ARG4*-telomere (the *ARG4*-containing sequence contains an ARS). The constructs were ligated to opposite ends of the insert (liberated by *Not*I digestion) of a fosmid clone and the resulting vector transformed into *C. albicans* strain 1006. Neither the vectors alone nor most fosmid inserts were stable (<15% stability per mitosis [including integration into the chromosomes]), but one 40 kb DNA fragment showed 92% stability and independence of the chromosomes under the same conditions. The region of the clone essential for stability is being determined. This fosmid maps to the region of chromosome 7 to which the centromere has been localized. This work was supported by USPHS Grant AI 16567 awarded to P.T.M.

# ASSESSING THE ROLES OF THE a1 AND α2 REPRESSOR PROTEINS IN THE PATHOGENIC YEAST *CANDIDA ALBICANS*

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The properties of *Candida albicans* that allow its colonization have been difficult to study because *C. albicans* is a diploid organism with no known sexual cycle. This asexual state severely hinders genetic analysis. Nevertheless, features such as adhesiveness, protease secretion and morphological switching have been implicated in pathogenesis. Some of these functions have clear parallels in the sexual functions of other fungi, and an hypothesis has emerged which suggests that functions used by ancestors of *C. albicans* for mating are now used by *C. albicans* to enhance pathogenesis. The pheromone response pathway identified in the related yeast *Saccharomyces cerevisiae* may be one of the molecular pathways altered for pathogenesis function in *C. albicans*. A gene was identified previously in *C. albicans* (*CAG1*) that is homologous to the *S. cerevisiae* gene encoding the Gα subunit of the trimeric G protein complex involved in signaling downstream events in the mating pheromone response pathway (*GPA1*). Interestingly, *CAG1* contains a conserved binding site for the regulatory proteins a1 and α2. In *S. cerevisiae*, the a1 and α2 proteins are involved in repressing the transcription of genes specific to haploid cells, so it is intriguing that a diploid organism like *C. albicans* has conserved a1/α2 binding sites in light of the fact that no haploid state has been detected. The presence of the sites is even more curious given the fact that no a1 or α2 homologs have been identified, and the *CAG1* shows no transcriptional regulation in *C. albicans*. The goal of this project is to explore the possibility that a1- and α2-like proteins may regulate genes involved in *C. albicans* pathogenesis or an as yet undetected sexual cycle. Toward this goal, extracts from *C. albicans* were tested for a1 and α2-like binding activities. An activity has been identified in *C. albicans* extracts that binds to an a1/α2 site in vitro, and its binding properties have been characterized. In addition, the *S. cerevisiae* proteins a1 and α2 have been expressed constitutively in *C. albicans*. The proteins have been shown to be competent for DNA binding, and preliminary results suggest that *S. cerevisiae* a1 and α2 mediate repression in *C. albicans*. An investigation of the regulatory role of the a1 and α2 proteins in *C. albicans* is underway.

# A SINGLE GENE FROM *CANDIDA ALBICANS,* EXPRESSED IN *S. CEREVISIAE,* IS SUFFICIENT TO INDUCE ELONGATED GERM TUBES AND ADHESION TO EPITHELIAL CELLS

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*INT1* is a *C. albicans* gene isolated by virtue of its limited homology with vertebrate integrins. We are using the budding yeast *S. cerevisiae* to study the molecular properties of Int1p. We show that expression of *INT1* in *S. cerevisiae* is sufficient 1) to direct a morphologic switch to a highly elongated filamentous form similar to *C. albicans* germ tubes[1] and 2) to endow yeast cells with the ability to adhere to mammalian epithelial cells. Immunoprecipitation of biotinylated surface proteins with antibodies specific to Int1p epitopes demonstrates that Int1p is present on the exterior surface of *S. cerevisiae* cells expressing *INT1*. *INT1* appears to mediate both adhesion and morphogenesis in *C. albicans* as well. Disruption of both copies of *INT1* in *C. albicans* suppresses hyphal growth on solid medium; and reintegration of *INT1* into its native chromosomal locus restores the hyphal growth phenotype. Disruption of *INT1* in *C. albicans* also reduces the level of specific adhesion to epithelial cells. Thus, a single surface protein, Int1p, is sufficient to induce both morphologic switching and epithelial adhesion in *S. cerevisiae* and is involved in these processed in *C. albicans* as well.

Interestingly, *INT1* expression induces filamentous growth in yeast on rich medium and in the absence of *STE20*, a component of the *S. cerevisiae* pheromone response pathway that is required for pseudohyphal growth[2] in response to starvation conditions, but is not required for serum-induced hyphal growth[3]. We are currently exploring the genetic requirements, cell cycle restrictions and morphologic characteristics of *INT1*-induced filamentous growth. We have explored the relationship between Int1p and Bud4p, a protein involved in bud site selection in *S. cerevisiae,* because these proteins share limited homology. Int1p is not a functional homolog of Bud4p: Int1p induces germ tubes in *bud4Æ* strains and overexpression of Bud4p does not give rise to elongated cells. We have analyzed the formation of Int1p-induced germ tubes during the cell cycle and find that cells must pass START to form germtubes, indicating that Int1p-induced filaments are highly polarized buds.

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DEVELOPMENT OF A DOMINANT SELECTION MARKER FOR STUDIES ON PUTATIVE VIRULENCE GENES OF *CANDIDA ALBICANS.*

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Progress in molecular genetics of the opportunistic pathogen *Candida albicans* has been hampered by the absence of a haploid phase of this fungus and the unusual decoding of the CUG codon to serine instead of leucine. All selection markers used for *C. albicans* depend on auxotrophic strains which are very tedious to generate in a diploid organism and therefore only a few strains are available for genetic manipulation.
We developed a dominant selection system for *C. albicans* based on inosine monophosphate (IMP) dehydrogenase and the specific inhibitor mycophenolic acid (MPA). IMP dehydrogenase is the key enzyme in *de novo* biosynthesis of guanosine monophosphate (GMP). It catalyzes the NAD-dependent oxidation of IMP to xanthosine monophosphate (XMP). The IMP dehydrogenase gene of *C. albicans* (*IMH3*) was cloned and sequenced. Overexpression of *IMH3* confered resistance to MPA and allowed selection for transformants (Köhler, G. A., T. C. White, and N. Agabian. 1997. J. Bacteriol. 179:2331-2338). Now we present an improved selection system using a mutant form of the *IMH3* marker which enables us to select for a single copy of the marker gene without the need of overexpression or auxotrophy. Gene replacement studies of putative virulence genes in wild-type strains of *C. albicans* will be shown to demonstrate the applicability of the new selection system.

## *CRYPTOCOCCUS NEOFORMANS* DIFFERENTLY REGULATES B7-1 (CD80) AD B7-2 (CD86) EXPRESSION ON HUMAN MONOCYTES

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To induce a specific response in primary resting T cells two signals must be provided by antigen presenting cells (APC). The first antigen-specific signal is mediated by formation of the T cell receptor major histocompatibility complex molecule ternary complexes. The second signal is delivered by interaction of either B7-1 or B7-2 expressed by APC with CD28 or CTLA-4 on T cells. In this study the modulation of B7-1 and B7-2 molecules on human monocytes pulsed with encapsulated or acapsular *Cryptococcus neoformans* or *Candida albicans* is examined. In our experimental system *C. albicans* or acapsular *C. neoformans* are able to induce B7-1 expression while the encapsulated yeast is a poor stimulator of B7-1 molecule expression. A modest increase of B7-2 expression was also observed after monocyte treatment of acapsular *C. neoformans* or *C. albicans*, while the encapsulated yeast was ineffective in inducing B7-2 molecules. Kinetic analysis showed that the maximum expression of B7-1 was revealed after 24 to 48 h of incubation while a substantial decrease was observed after 72 h. The contribution of B7-1 and B7-2 costimulatory molecules to cryptococcal-specific T cell activation was analyzed and a substantial inhibition of T cell proliferation was observed. In this study we provide the first demonstration of fungal interference with costimulatory molecules on APC and a role for capsular material of *C. neoformans* in regulating CS molecules.

## PROBING THE PARASITIC LIFESTYLE OF *HISTOPLASMA CAPSULATUM*

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*Histoplasma capsulatum* is a dimorphic fungal pathogen that grows in a saprophytic mycelial form or a parasitic yeast form. In its yeast phase, the organism is superbly adapted for survival and proliferation within mammalian cells. We have demonstrated that *H. capsulatum* is able to survive and multiply in a phagolysosome, maintaining this compartment at a near-neutral pH to avoid enzymatic destruction. These studies have focused on survival within macrophages, but we have also isolated a series of spontaneous *H. capsulatum* variants that grow poorly within macrophages yet proliferate readily within respiratory epithelial cells. In order to understand the genetic basis of this switch in host cell preference and other virulence-related phenotypes, we have developed a telomeric shuttle vector system that allows maintenance of cloned DNA as a linear extrachromosomal plasmid in *H. capsulatum.* We have also constructed similar telomeric plasmids with Histoplasma promoter regions fused to lacZ, allowing us to monitor easily the activity of cloned genes in a Histoplasma genetic background. This approach is currently being used to evaluate the regulation of CBP1, which encodes a major secreted protein that appears to be involved in calcium uptake. In addition to using these plasmid systems, we have also been examining how randomly integrating DNA can be exploited as a mutagenesis tool. This is an adaptation of restriction enzyme-mediated integration (REMI), which promotes restriction site-specific insertion events in the recipient genome. All of these tools are currently being employed to identify and characterize structural and regulatory genes involved in the pathogenesis of histoplasmosis.

IMMUNOREACTIVE EPITOPES OF *COCCIDIOIDES IMMITIS* RECOMBINANT ANTIGENS.

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Antigen 2 (Ag2), a glycosylated protein present in mycelial and spherule-phase cell walls of *Coccidioides immitis*, is believed to be a major T cell reactive component of this dimorphic fungus. Ag2 was cloned using a cDNA expression library derived from spherule-phase mRNA and the clone was shown to contain a 582-bp ORF which encodes for a protein comprised of 194 amino acids. Ag2 cDNA was ligated to the pGEX-4T-3 vector and expressed as a glutathione *S*-transferase (GST) fusion peptide in *E. coli*. The recombinant Ag2-GST (rAg2) peptide yielded double bands, having molecular sizes of 55 and 57 kDa, in immunoblots probed with sera from coccidioidomycosis patients, but was without reactivity with sera from histoplasmosis or blastomycosis patients. To evaluate the location of B-cell reactive epitopes, PCR-generated Ag2 truncations were used as target antigens in enzyme-linked immunosorbent and immunoblot assays of sera from patients with various stages of coccidioidomycosis. Both linear and conformational B-cell reactive epitopes were shown to be localized within a domain comprised of amino acids 19 through 96. Antibody reactivity directly correlated with disease severity. Whereas patients with pulmonary disease showed a mean response of $0.16 \pm 0.04$ ($A_{405nm}$), patients with disseminated coccidioidomycosis showed a mean response of $0.69 \pm 0.17$ ($p<0.05$). The T cell reactivity of rAg2 was evaluated by delayed-type footpad hypersensitivity tests in *Coccidioides* immune mice. Ag2-GST elicited significant footpad hypersensitivity responses, with a mean of $27.0 \pm 6.0$ ($\times 10^{-2}$ mm) as compared to $4.1 \pm 2.2$ in mice tested with GST alone ($p<0.0001$). Additionally, rAg2 induced expression of the T-helper 1 (Th1)-associated cytokine IFN-$\gamma$ in *Coccidioides* immune but not nonimmune mice. Since T-helper 1 cell reactivity strongly correlates with resistance to *C. immitis*, studies were done to determine if the rAg2 peptide might induce protection in mice. Intra-muscular injection of BALB/ mice with rAg2-GST engendered protection against intraperitoneal challenge with 250 arthroconidia. The preceding results, taken together, document the utility of rAg2 as a tool for evaluating T and B cell responses in coccidioidomycosis and as a potential vaccine for this disease.

## THE SEARCH FOR A VACCINE FOR COCCIDIOIDOMYCOSIS

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Coccidioidomycosis (cocci) is a disease caused by the pathogenic fungus Coccidioides immitis. Cocci is an important disease in the southwestern U.S. Though 95% of the cases of cocci resolve spontaneously, resolution can take months and in 5% extra-pulmonary dissemination occurs. Since cocci confers lifelong immunity, a vaccine is a feasible goal. T-cell mediated, rather than humoral immunity is required for protective immunity. We have tried to identify T-cell immunogens in C. immitis, using a mouse model of infection. We have cloned and sequenced a C. immitis T-cell reactive protein (tcrP). This protein was identified because it stimulated a mouse C. immitis-specific T-cell line. TcrP is homologous to the enzyme 4-hydroxyphenyl-pyruvate dioxygenase found in a wide variety of species including mice and human beings. TcrP is found in the wall of the C. immitis spherule. TcrP, expressed in E. coli, elicits good T-cell proliferation in lymph node T cells from C. immitis-immune mice. The tcrP response T-cells produce interferon-$\gamma$. Immunization of susceptible mice with recombinant tcrP affords a tenfold reduction in fungal burden after intraperitoneal challenge. This is the first example of a recombinant protein providing a partially protective immune response to cocci in mice.

ZINC DEPRIVATION, ANOTHER SIGNAL TRIGGERING *Aspergillus* GENE EXPRESSION?

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Several *Aspergillus* species are opportunistic pathogens causing respiratory diseases in normal hosts and invasive or disseminated infections in immunosupressed patients.

ASPND1 is an *Aspergillus nidulans* immunodominant antigen that is highly reactive to sera from Aspergilloma affected individuals. Molecular cloning and characterization of its encoding gene (*aspnd1*)(1) revealed a high degree of homology to a well characterized *A.fumigatus* allergen (ASPF2), consistently reactive to sera from Allergic Bronchopulmonary Aspergillosis cases (2). Both cross-reactive antigens (of hitherto unknown function) induce a strong immune response in humans and have been overproduced in bacteria as recombinant proteins, retaining their ability to react with both IgG and IgE specific antibodies. A common characteristic of both antigens is that they are only detected when the fungi are grown in certain conditions (3). These observations may reflect how the expression of these proteins is regulated "in vivo" and could provide some clues about their function, if any, as determinants of virulence.

In bacterial systems, virulence genes seem to be integrated into complex regulatory networks which determine the expression of virulence factors only when needed. In most cases these regulatory circuits switch on in response to a few key environmental signals such as a temperature of 37°C, iron deprivation or contact with eukaryotic cells, all of them features of the inside of the body of the mammals (4,5). In the case of *Aspergillus*, only iron deprivation has been shown to stimulate the synthesis of products involved in the survival of the invading fungus (6).

We report here that the factor responsible for the production of ASPND1 and ASPF2 production is a lack of $Zn^{2+}$ in the culture medium. We have found identical regulatory mechanisms in *A.fumigatus* and other *Aspergillus* species. We offer the first evidence that Zinc deprivation can induce the "in vivo" synthesis of specific Zinc-regulated fungal proteins (ZRPs) in the human body. Zinc starvation could consequently be considered as a new signal for fungal pathogens from the host environment.

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# THE SYMBIOSIS OF *AEROMONAS VERONII* AND *HIRUDO MEDICINALIS*, A NOVEL ANIMAL MODEL

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In the study of pathogenesis, animal models are of fundamental importance for the identification of virulence factors. An alternative approach to traditional vertebrate models are naturally occurring, nonpathogenic associations with invertebrates. For many years, *Aeromonas* species have been implicated in human diseases ranging from enteritis to septicemia. Yet the identification of virulence factors has been hampered by the lack of appropriate animal models. A promising alternative is the mutualistic association between *Aeromonas* and the medical leech, *Hirudo medicinalis*. The extracellular symbionts colonize the digestive tract, proliferate rapidly upon the ingestion of blood and are thought to aid in its digestion. We identified the symbionts as *Aeromonas veronii* biotype *sobria*, a biotype that has been associated with human disease, including diarrhea and septicemia. Symbionts of the leeches applied to patients postoperatively frequently cause septicemia unless antibiotics are administered. Thus the same strain appears to be pathogenic in humans and mutualistic in the medical leech. Genetic analysis of these strains may provide an excellent opportunity to identify factors important for host colonization, proliferation in blood and may shed light on the evolution of virulence. Using plasmid carrying transconjugants of a symbiotic isolate, we were able to select for genetically manipulated strains inside the digestive tract of the leech. This approach allowed the quantitative recovery of the bacteria and evaluation of the specificity of the symbiosis. The expression of the RpoN regulated *tapA* promoter and autoregulated promoters of the *luxRI* homologs were tested *in vivo* with the green fluorescent protein as a reporter gene. The mutualistic association of *Aeromonas* with the medical leech, combined with the use of *gfp*, allows for the identification of active promoters during the colonization process and thus will provide a window into this symbiosis and perhaps pathogenesis.

# IDENTIFICATION OF NOVEL *PSEUDOMONAS AERUGINOSA* VIRULENCE FACTORS USING PLANTS AS MODEL HOSTS

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We have used plants as an in vivo pathogenesis model system for the identification of novel virulence factors of the human opportunistic pathogen Pseudomonas aeruginosa. Following transposon TnphoA mutagenesis, we identified ten mutant derivatives of *P. aeruginosa* strain UCBPP-PA14 that are significantly reduced in plant pathogenicity. All ten mutants also exhibited significantly reduced virulence in a mouse model suggesting that bacterial pathogens use many common strategies to infect both plants and mammals. DNA sequence analysis showed that eight of these mutants contain TnphoA insertions in novel genes. Two mutants contain insertions in known genes previously identified as virulence factors of both mammalian and plant bacterial pathogens. These results demonstrate that an alternative non-vertebrate host of a human bacterial pathogen can be used in an in vivo high throughput screen to identify previously unknown bacterial virulence factors involved in mammalian pathogenesis.

*MYCOBACTERIUM MARINUM* AND GOLDFISH, *CARASSIUS AURATUS,* A MODEL SYSTEM FOR MYCOBACTERIAL PATHOGENESIS. A. Talaat, R. Reimschuessel and M. Trucksis. University of Maryland School of Medicine, Baltimore, MD 21201.

We have developed an animal model for studying *M. marinum* using the goldfish, *Carassius auratus*. Using this model goldfish are injected intraperitoneally with doses between $10^3$ and $10^9$ colony forming units (cfu) of *M. marinum*. Depending on the dose of *M. marinum* administered an acute or chronic disease is produced. The acute disease (induced by $10^9$ and $10^8$) is characterized by systemic mycobacterial infection, severe peritonitis, necrosis of organs, and death within 17 days of infection. The chronic disease is characterized by survival of all animals to the endpoint of the experiment (56 days) with formation of granulomata in all organs. Granulomata were present in fish infected with as little as $10^3$ cfu. The colony counts from organs in the chronic disease group ($10^7$ inoculum) showed a decrease in numbers in 2 of 3 organs evaluated at 8 weeks suggesting systemic immunity may be developing with clearance of the mycobacteria. We believe this well characterized animal model will be useful for studying mycobacterial pathogenesis.

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